Protocols for methods of field sampling

Deliverable D1.1

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Foreword

The PoshBee field study involved nine research partners based in eight European countries: Ireland, UK, Spain, Germany, Sweden, Estonia, Switzerland and Italy. The aim of the study was to gain a holistic picture of the multiple environmental and biotic stressors encountered by bees occurring in representative agricultural landscapes in each of the participating countries. To meet this aim a full suite of measurements was planned to encapsulate the variation in habitat quality, the abundance of flowering plants, the diversity of chemical pollutants present on the bees themselves and in their food resources, and the frequency and co-occurrence of colony pests and pathogens, that bees encountered under typical field conditions.

In practice, the above processes required the establishment of three species of commercially produced 'sentinel' bees (*Apis mellifera, Bombus terrestris, Osmia bicornis*) within two mass-flowering crops, apples and oilseed rape, in all eight countries. But, given the relatively short flowering time of these crops, the desire for high levels of replication (16 sites per country), and the natural sequence that would occur in flowering times across the study network, standardisation of timings, various habitat assessments and insect sampling methodologies required some considerable effort. The study design and methodological protocols summarized in this document arose from many months of meetings and online discussions among the field study partners, with regular input from other groups in the PoshBee project that would be involved in processing the samples we obtained. Ultimately, many compromises were made: for example between research groups with long-established, but different, pollinator sampling methods, and between the amounts of material requested by analytical laboratories and the amounts of material that were feasible to collect in the time available.

These documents were produced to enable standardized sampling, and so do not adhere to a strict formatting code and were not written in typical prose. In general, each protocol consists of a series of bullet points, and, where appropriate, templates for the collection of data in order to encourage consistency in the final results. Because several measurements and samples were collected on the same visit to a study site, many of the protocols include cross-referencing to other protocols (using the document codes WPx.xx) to highlight these links. Clearly, uses of specific contact names and postage addresses are only relevant to the PoshBee project, and thus have been removed from this document. Because these documents were prepared for 'in house' use, our use of citations is more functional than standardized among the documents, and often bibliographical details of information sources are given at the end of each document. Nevertheless, we have endeavoured to recognise all sources of information, have added references to online material where relevant images and videos can be found, and credit image sources.

Simon Hodge & Jane Stout, 2020

WP1.1.1 Field site selection

O Schweiger, S Hodge, M Rundlof, C Dominik

11.02.2019

General

- In each country, the field site network will consist of **EIGHT** sites of each of **TWO** crops.
    - Winter-sown oilseed rape fields (OSR; *Brassica napus*)
    - Apple orchards

- Preference should be given to sites:
    - located close to home institutions or within a particular region to reduce travel time.
    - where background information exists on surrounding habitat, land cover, land-use, intensity, existing vegetation survey data or data on managed bees or wild bees, etc.
    - where established contacts to local farmers already exist.
    - close to where the volunteer beekeepers are based.

- A site is defined by the individual **OSR field** or **apple orchard** which contains the sentinel hives/colonies.

- For statistical purposes, much of the habitat, chemical, -omics, bee health data, and grower questionnaire data etc will be summarized on a **site basis.**

- Detailed landscape data (WP1; WP8) will be collected over a circular area with a **1 km radius** centred on the sentinel hives (see WP1.3.1, WP1.3.2).

- There is **NO** requirement to select or reject sites on the basis of **longitude, latitude, altitude** or **aspect**. These components, which could influence climate and local weather and soil conditions, will be recorded and included in statistical models.

- Ideally (for statistical independence of sites based on known foraging distances of the sentinel bee species) sites should be at least **6 km apart**. However, if this is not feasible, sites should be spaced as far apart as is possible.

- There is **no minimum** area of target crop, as field/orchard size will vary among the different countries. If possible, 'typical' sized fields/ orchards of each country should be selected.
  The area of the target field/orchard will be recorded and included in the data set.

- The sentinel hives/ nests will be placed along one field **boundary** (see WP1.2.4).

- The class of vegetation in the fields **adjacent** to the target field will also be recorded (e.g. grass/pasture; arable; orchard; semi-natural) as this will directly affect bee resource availability (see WP1.3.1).

- For each crop and management system, it is desirable to select sites that provide variation in resource availability to the bees and surrounding habitat type (e.g. habitat diversity; agricultural intensification; agricultural diversity; semi-natural habitat etc). For details for each crop see below.

APPLES

For apples, **WITHIN EACH COUNTRY** we aim to select apple sites representing a gradient of agrochemical use. This could be achieved by selecting gradients based on:

- prior knowledge of grower's chemical use (e.g. organic v IPM v conventional farmers)

- the proportion of apple orchards in the surrounding fields
- the proportion of all orchards and arable land/ annual crops in the surrounding fields
- the proportion of all agricultural land use in surrounding fields (use GIS/ Google maps etc)
- the proportion of semi-natural habitat in surrounding fields

Selection procedures based on the proportions of orchards, agricultural land and semi-natural habitat in the surrounding area are founded on using these land types as proxies for describing pesticide exposure to the sentinel bees.

For example,

- In the highest spectrum of the agrochemical use gradient (see figure 1), try to select conventional or integrated pest management sites, primarily reliant on synthetic pesticides/ fungicides, high usage of inorganic fertilizers and surrounded by mostly other orchards and arable land with little semi-natural habitat or woodlands.
- In the lowest spectrum of the agrochemical use gradient (see figure 1), try to select sites that are more traditional apple production methods, certified organic or 'chemical free', where there is no (or infrequent) use of synthetic biocides and soil nutrients are maintained by use of manure or organically-derived products (seaweed; fish waste; animal products). These sites should be surrounded by few other orchards or agricultural land and be close to semi-natural habitat and woodlands.

In general, site selectors in each country are free to choose sites based on their own criteria in terms of agro-chemical inputs, as this will vary among countries, as will the range of products, and production systems used.

However:

- All orchards must be **COMMERCIAL** orchards
- Orchards with table/ desert/ cooking apple varieties can all be used.
  Orchards with cider apple varieties should only be used if no other suitable sites can be found.
- Ideally, use the same **VARIETIES** of apples for both high input and low input. If this is not possible use varieties in pollination groups which have similar **FLOWERING PERIODS** (in your country)

Selection procedure for apple sites

- Identify apple orchards (with the help of your farmers' organisation; be aware of the travelling time) and get latitude and longitude coordinates of the centre points (in decimal degrees, WGS84).
- Select a pool of candidate sites along a gradient of the amount of area in the surrounding landscapes covered by apple orchards. If this is not possible, use the total area of orchards and arable land as a proxy, or even the proportion of agricultural land. You may use national geo-databases, aerial photographs or google maps. You may also send the coordinates (WGS84, latitude longitude in decimal degrees) to UFZ where the amount of agricultural area, arable land, orchard, grassland and forest will be extracted and provided to you for site selection. The resolution will be 5 km. This land use information may only serve as a first indication and ground-truthing may still be needed.
- Up to **two organic orchards** may be used as representative of the lowest input systems along the agrochemical use gradient.
- Sites that have **no or low chemical** input but are not certified organic can also be used to help create the agrochemical gradient
- Start with eight sites that are spread more or less evenly across the intensity gradient (amount of orchards and arable fields) and apply all relevant selection criteria, and have the agreements of the land owners and identify that they are amenable to beekeepers. Visit the sites and check all requirements.
- If one or more sites do not match all requirements, go back to the candidate list and repeat the procedure to fill gaps.

<u>OILSEED RAPE</u>

For winter-sown OSR, treatment with agrochemicals may not differ much within a country (or OSR growing region). At least some variation may occur due to site-specific management practices of individual growers, although this may

be difficult to anticipate prior to contacting the grower. We will (hopefully) find a more pronounced gradient of agrochemical use across Europe.

Given the situation described above, where possible, site selectors should try to obtain OSR sites that represent a **gradient of agrochemical use (IN THEIR COUNTRY)** (see figure 1)**.** This could be achieved by selecting gradients based on:

- prior knowledge of growers chemical use (e.g. organic v IPM v conventional farmers)
- the proportion of OSR in the surrounding fields
- the proportion of arable land (i.e. annual crops) in the surrounding fields
- the proportion of agricultural land use in surrounding fields (use GIS/ Google maps etc)
- the proportion of semi natural habitat in surrounding fields
- For OSR, there is **NO** need to restrict site selection based on:
    - Variety/ cultivar
    - Field size [although it has been suggested a minimum of 1 ha should be aimed for]

Selection procedure for OSR sites

- With the help of your farmers' organisation, select a region where OSR is grown (hopefully not too far from your institute and/ or close to the apple sites).
- Select a pool of candidate sites along a gradient of the potential amount of area covered by rape. If this is not possible, use the total area of arable land as a proxy. You may use national geo-databases, aerial photographs or google maps.
- You may send the coordinates (WGS84, latitude longitude in decimal degrees) to UFZ, where the amount of agricultural area will be extracted and provided to you. The resolution will be **5 km** to increase the probability to have rape fields within the grid cell: this might only serve as a first indication and ground-truthing would still be needed.
- Start with eight sites that are spread more or less evenly across the intensity gradient (amount of rape or of arable fields) and apply all relevant selection criteria (as mentioned, above plus potentially needed agreements with farmers and beekeepers).
- If these are present in your country, up to **two organically managed** oilseed rape fields may be used as representative of the lowest input systems along the agrochemical use gradient.
- Farmers who indicate they use low chemical inputs or IPM strategies for pest control may also help create your agrichemical gradient.
- If your sites do not produce the required gradient go back to the candidate list and repeat the procedure to try and fill gaps.

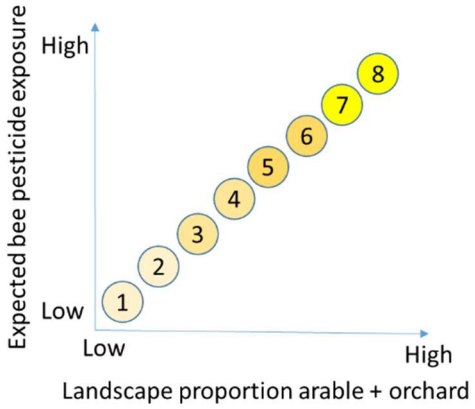

Figure 1. Schematic diagram of the eight sites of each crop located along a management intensity gradient, indicating the proportion of intensively used agricultural land in the surrounding landscapes and the predicted level of bee exposure to pesticides.

WP1.1.2 Site labelling scheme

O Schweiger, S Hodge

06.03.2019

Throughout the WP1 field survey, all data and samples must be labelled in a consistent manner that identifies the country, crop and site, using the format below:

ThreeLetterCountryCode_ThreeLetterCropCode_TwoNumberSiteCode

Country codes (ISO 3100-1 alpha-3)

Country	Code
Estonia	EST
Germany	GER
Ireland	IRL
Italy	ITA
Spain	ESP
Sweden	SWE
Switzerland	CHE
United Kingdom	GBR

Crop codes

Crop	Code
Oilseed rape	OSR
Apple	APP

Site codes

Use a unique **two-number site code** this is so that NO information related to the geographic location, the owner, farmer, organisation, producer name etc. is involved in the labelling.

The number codes should run from 0**1-16** for each country: **01-08 for OSR** ; **09-16 for apples**.

Examples

"GER_OSR_03"

"IRL_APP_13"

Species / Hive / nest / colony codes

The three bee species can be labelled: A - *Apis*; B - *Bombus*; O -*Osmia*

The three *Apis* hives, *Osmia* trap nests and *Bombus* colonies should be given code numbers so that data or notes relating to specific hives/ nests can be related easily.

Apis hives should be labelled at each site as: A1, A2, A3

Bombus colonies should be labelled at each site as: B1, B2, B3

Osmia nests should be labelled at each site as: O1, O2, O3

Labels can be extended as:

"IRL_APP_13_O1" "IRL_APP_13_A3"

SUBSTANCE codes: Pollen / beebread / pollen stores

For the pollen extracted from flowers:	FP
Pollen from *Apis* pollen traps:	AP
Pollen from *Bombus* legs:	BP
Beebread from *Apis* hives:	APS
Pollen stores from *Bombus*:	BPS
Pollen stores from *Osmia*:	OPS

eg "IRL_APP_13_FP" eg "IRL_APP_13_OPS"

Dates

Dates should be written in standard EU format DD-MM-YYYY 26-03-2019

All samples taken from the field and stored in jars/bags must be labelled with:
COUNTRY CODE, CROP CODE, SITE CODE, SPECIES CODE or SUBSTANCE CODE and SAMPLING DATE.
Individual HIVE/NEST codes may replace the SPECIES CODE where necessary.

For example, *Bombus* from Spanish OSR site 8:	ESP_OSR_08_B_01-04-2019
Bombus from Spanish OSR site 8 and *Bombus* nest 1:	ESP_OSR_08_B1_01-04-2019

Collection of bees:

An additional label will be added for the samples of bees according to the type of analyses planned.

Bees will be sampled on the field and stored in jars until return to the field labs. Then samples will be pooled per species and sub-divided according to the destination of the different labs.

The bees used for haemolymph analysis will be stored in the pre-prepared labelled tubes provided by the proteomics team (WP1.6.1).

For the other samples, to ease recognition of samples arriving to the field labs and postage, the labels of the different jars should distinguish the purpose of the sample.

So, the labels of the jars should be extended as follows:

ANALYSIS codes:

- Nectar collection and pesticide residue analyses: NTOX
- Pesticide residue analyses for *Osmia*: TOX
- Metal analyses for *Apis* foragers: MET
- Pathogens analysis: PAT
- Wing asymmetry analyses: WA
- Gut microbiota GUT

For example, a jar containing *Apis* foragers sampled in the field from hive 1 for metal analyses should be labelled as follows:

ESP_OSR_08_A1_MET_16-04-2019

For example, a jar containing pooled *Bombus* foragers used for nectar collection and then pesticide residue analyses should be labelled as follows:

ESP_OSR_08_B_NTOX_16-04-2019.

WP1.1.3 Basic site data

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11.02.2019

General site data

1. To initiate the formation of WP1 databases, we require basic data for each site in each country.
2. Some of these data could be collected prior to the start of the field season or by asking the grower.
3. Further data will be added to these databases as the project progresses and more variables are measured.
4. These data will provide the basis for analysis of response variables measured in other work packages (eg WP2 and WP9)
5. Be mindful of GDPR issues. No personal information pertaining to the growers, or information that could identify them directly, needs to be stored on these central databases.
6. The data for flowering dates can be supplied separately once this information is known.
7. Record these data on the tables provided (or associated Excel sheet).

For all sites, record:

- Site label (see WP1.1.2)
- Latitude; Longitude (use location where the central *Apis* hive will be positioned; 6 decimal places; 53.330201, -6.220260)
- Area of target crop as provided by the grower (ha or m^2)
- Date flowering started, defined as date first flower appeared, as provided by the grower (DDMMYY).
- Date flowering ended (DDMMYY) as provided by the grower.
- When *Apis* hives, *Bombus* colonies and *Osmia* nests were installed/ removed

For Apples

Variety.	The dominant variety near the focal site
Diversity.	The number of varieties grown in the orchard.
Density.	By quick field survey (stems per ha).
Height.	By quick estimation (m)
High/Low input system	Description provided by the grower

Oil Seed Rape

Variety	Provided by the grower
Plant density	By quick field survey (plants per m^2).
Date sown	Provided by the grower (DDMMYY).

WP1.1.4 The WP1 Data Management Plan

S Hodge

11.02.2020

General PoshBee Data Management

1. An overall data management plan for the PoshBee project will be developed as part of WP11.4 which will cover data storage, access, audit trails, and so on.
2. GDPR issues are covered by the requirements of WP13
3. ANSES and other WP2 partners will develop an 'online centralised database' that will be accessible by all partners and eventually be open for consultation by policymakers.
4. This document is related to the collection, collation, security and risk mitigation of data collected during the **WP1 field surveys**
5. Within the WP1 site network, we will produce:
   - large quantities of biological and landscape data
   - substantial amounts of meta-data relating to the samples collected for WP2 and WP9
6. This information will be collated and stored in a standardized format where it can be accessed by PoshBee partners.
7. With a view to future data sharing, some of the data and information we collect will later be made open access and stored in data repositories where it can be accessed and used by all interested parties.
8. The ultimate aim is to produce high-quality, well-maintained data files that simplify subsequent statistical analysis, and promote meaningful interpretation.
   The data sets should be comprehensible to the researchers who helped supply the data within them.
   The data sets should be amenable to fostering analysis and potential cross-collaboration in the future.
9. It is desirable that we consider data collection, storage, manipulation and security **prior** to obtaining field measurements and sample collection
10. We must be aware of **GDPR** issues associated with any personal data we retain, and who is allowed to, or needs to, access such data.
11. Developing efficient collection, storage and analysis strategies in advance of performing the field survey will optimize the use of the data we collect. Be mindful that this data management will require some allocation of resources by each group: human resources, electronic/digital storage and the maintenance of audit trails (eg hard copy → digital format → corrections → final versions).
12. **Do NOT assume** someone/ everyone in your group is taking care of data management; if possible a specific person should be appointed or take responsibility for your WP1 data management issues.

Data collection – points to consider when designing sampling protocols

1. To ensure consistency in data collection across the WP1 field network; all the standardized WP1 sampling protocols should be associated with a template for standardized data collection.
2. Data templates should also include spaces to include meta-data associated with each sampling event (e.g. collection date; time; personnel involved).
3. Data collection templates should be linked to instructions regarding what measurements need to be taken, the units of measurement, and the form of data that should be recorded (e.g. counts; codes; presence / absence; continuous measurements; decimal places to be used etc). The more detail provided the better.
4. When designing the sampling protocol be mindful of what data are actually required to answer the purpose of that aspect of the research.
   - Will the data obtained be fit for purpose?
   - Could the data collection / sampling process be simplified and still be fit for purpose?
   - Is there a risk associated with recording only simplistic low precision data that is quick to obtain? (e.g. scoring presence/absence of insects/pests rather than taking actual insect counts)
5. Also consider whether:
   - the data collected can be converted into useful, easily comprehensible information
   - can the data be converted into concepts that are easily understood by the general public (e.g. $; deaths; kg food; risk of extinction)?
   - will the data collected produce suitable outputs for the various stakeholders involved in the

project? (e.g. scientists; farmers; policy makers; general public).
- can the same data be used to meet multiple end points?

Practicalities for WP1

1. In WP1 we aim to:
   - record standardized data across the whole site network
   - minimize risk of data errors
   - minimize risk of data loss (human error; theft; Acts of God!)
2. Use prepared data sheets for recording data in the field.
   - Data sheets should be supplied with each field protocol.
   - Data sheets should explain what format / units the data should be obtained.
   - An Excel sheet/Word document of the same data sheet should also be distributed.
3. We aim to use consistent coding over the whole network
   - The coding to be used should be explained in each sampling protocol.
   - For example, the standard site code format has been explained in **WP1.1.2**
   - The coding of field boundaries within each site is explained in **WP1.3.1**
4. In the field, data can be added to data sheets as hard copy (eg lab books / note books / clip boards) or directly into digital form (tablets / iPads / laptops).
   - In both cases, errors could be avoided if data sheets are pre-prepared, labelled, meta data completed etc prior to going into the field.
5. - If hard copy data sheets are used, sheets should be photographed using a digital camera or mobile phone as instant back up.
   - Use waterproof ink pens or pencils to record the data
   - Data sheets should be stored in a folder or ring binder in plastic covers.
   - Data should be entered into Excel spreadsheets as soon as possible when the sampling is still fresh in the memory and any potential errors can be identified more readily.
6. If data are entered directly into spread sheets care should be taken to use unique file names to avoid saving over previous files.
   - Make sure meta data are changed between files where necessary.
   - Files should be backed up in the field using an external memory source or by email.
7. Use **sensible file names** for your data files so these can be located later (see below).
8. All raw data files and hard copies should be stored at the home institute for any data audits that are required and resolution of any future confusion.
9. Digital copies of all **raw data** should be sent to the **WP1** data coordinator at TCD (**Simon Hodge).** This will create an additional backup copy of all raw data at TCD for WP1 field data.
10. The data from the whole WP1 network will be collated at TCD.
11. Final versions of collated data files will be made available to all WP1 partners. The data relating to each WP1 partner should be checked for errors by the appropriate researchers.
12. Once final checks have been completed, these files will form the data master copies or ('Single Source of Truth; SST) to be used as the basis for future data analysis and data manipulation.
13. Final versions of collated files will be sent to the overall data management personnel for at (RHUL) additional storage and final processing, and to relevant people in WP2 and WP9.
14. Final data files will be made available to all PoshBee partners on the web page (poshbee.eu)

File Labelling

We need to use file names that are **simple, descriptive** and will **allow** other PoshBee workers to find the information they need easily

It is envisaged that all digital WP1 data files will consist of Excel files, possibly with multiple worksheets

Each partner should consider that when creating/ storing files then some reference to the WP protocol should be made, using the coding system below

When sending collated files to the WP1 data coordinator (SH), the following format should be used:

COUNTRY_PROTOCOL LETTER CODE_DATE MODIFIED

For example, the final version of the floral survey data from Ireland might be: IRL_FLOR_230419

Country codes (ISO 3100-1 alpha-3)

Country	Code
Estonia	EST
Germany	GER
Ireland	IRL
Italy	ITA
Spain	ESP
Sweden	SWE
Switzerland	CHE
United Kingdom	GBR

Examples of possible protocol letter codes

Protocol	WP1 Protocol code	Letter code
Basic site data	WP1.1.4	SIDA
Basic landscape data	WP1.3.1	LAND
Floral survey	WP1.3.3	FLOR
Wild bee counts	WP1.3.4	WILD
Interview growers	WP1.3.5	QUES
Pollen from target crop	WP1.4.2	POLL
Nectar from bees	WP1.4.3	NECT
Bees for WP2 chemicals / metals / health	WP1.4.4	WP2B
Beebread	WP1.4.5	BEEB
Colony strength	WP1.5.1	COLS
Apis - *Varroa*	WP1.5.2	VARR
Apis - Small Hive Beetle	WP1.5.3	SHB
Apis - *Vespa velutina*	WP1.5.4	VESP
Apis - chalkbrood	WP1.5.5	CHAL
Apis - American Foulbrood	WP1.5.6	AFB
Apis - European Foulbrood	WP1.5.7	EFB
Apis - Dwarf Wing Virus	WP1.5.8	DWV
Natural enemies - *Bombus*	WP1.5.9	BBNE
Natural enemies - *Osmia*	WP1.5.10	OSNE
Haemolymph	WP1.6.1	HAEM
Asymmetry + body fat	WP1.6.2	ASYM
Gut biota	WP1.6.2	GUTS

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WP1.2.1 Guidance for preparation of *Apis mellifera* colonies for 2019 field site network

J de Miranda, M-P Chauzat, C Costa, J Stout, S Hodge

04.03.2109

General

1. The main consideration should be to standardize the ***Apis*** colonies WITHIN each country.
   Aim for within-country uniformity in terms of boxes used, colony strength, genetic origin etc
   (e.g. each country should use local bees and local boxes)
2. The colonies must be created and maintained so they will be able to provide the various samples and data required for WP2.
3. In each **COUNTRY** we need 16 sites x 3 colonies = **48** viable colonies.
   Recommendation is to prepare 10-20% more colonies in 2018 to account for losses during winter 2018-2019, so that 50 colonies will be available for placement in spring 2019.
4. If possible, bees should be obtained from keepers known to apply good beekeeping practices.
5. The colonies should be on site 1-2 weeks before blooming starts to allow the colony to adapt to the surroundings, so consider time of blooming of apples and oil rape seed in your country to plan when colonies should be prepared.

BY THE START OF THE EXPERIMENT, THE COLONIES SHOULD…

1. … be free from diseases, or have 'typical' acceptable low level of pathogens and pests.
2. … have productive young queens (ideally 1 year old; 2 years max) from similar genetic origin.
3. … be of a normal strength for the season and location.
4. … be similar between **all** colonies for each crop (OSR, APP) in: overall size (adult bees and brood), brood composition (relative amounts of eggs, open brood and capped brood) and in food resources (honey, pollen).
5. … have an adult bee population that covers at least 7 to 10 frames, containing at least: 5-6 frames of brood, 2-3 frames of food resources, and 1-2 empty frames in order to allow for colony growth (see figure below).
6. If the colony is strong and forage is available, a box of drawn comb can be added, either as a second brood chamber or as a honey super, depending on the hive size and conventional practice for supering in your country. Whatever you do, apply it consistently for **ALL** colonies.
7. … be placed on site in the edge of the field 1-2 weeks before the start of the crop bloom at the particular site. Paint the entrances of the 3 hives with different colours to minimize drifting.

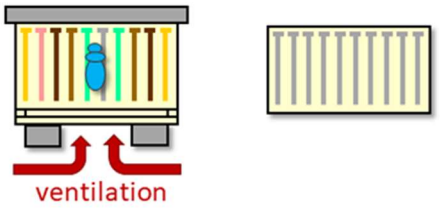
EQUALIZING COLONIES (2 weeks prior to placement)

1. In order to equalize the colonies prior to transport and placement, make a general inventory of all the colonies to be included in the experiment.
2. Re-distribute food resources, open brood and capped brood so that all colonies have about the same amount of these.
3. If there are large differences in **adult bees** between colonies, the safest way to equalize this d spring (i.e. now…) is to transfer more **capped brood** to the weaker colonies. These will emerge during the next two weeks to equalize the adult population by the start of the experiment.
4. Any further (inevitable) differences between the colonies will be accounted for with the official colony strength evaluation protocols **(WP1.5.1 Colony strength evaluation)**.

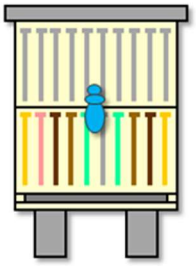
Honeybee colony
Field placement
(1-2 weeks before bloom)

honey	pollen	drawn comb
eggs	open brood	capped brood

A. Transport



B. Placement (1-2 weeks before bloom)



Summary of set up details

1. For POSHBEE site network, variations on the "Classical objective method" could be used if colonies are set up in spring 2019, while variations on the "Shook swarm method" could be enacted in summer 2018.
2. Preference for all bees to come from a single producer (to ensure uniformity), and queens should be of the same age and origin if possible.
   If this is not possible, use "Classical objective method" to mix and evenly distribute the bees and distribute the queens from different producers across locations.
3. Start colonies in July2018 using the "Shook swarm method" or as splits on drawn honeycomb with new queens (2018). Set up extra colonies to anticipate winter losses in your country.
4. Use the same kind of boxes within each country
5. If possible use residue–free foundations, and source brood combs from organic beekeepers to reduce risk of presence of acaricide residues in the wax.
6. Overwintered according to best beekeeping practices in each country.
7. All colonies should be subjected the same *varroa* treatment in each country, according to best beekeeping practices, aiming at minimizing mite loads for spring 2019.
8. At the beginning of blooming, colonies should be of a normal strength for the season and location (see Delaplane et al . 2013).

If possible

1. The colonies should have queens of the same age (1-2 years) and from the same mother origin.

2. Colonies should be homogeneous in size (adult bees and brood), in brood composition (about same number of young and capped larvae) and in food supply among treatments.
3. The colonies should be visited regularly, at least once or twice a week, for purposes of monitoring the health status and should be free of pathogens before the trial begins
4. Each colony should have a bee population that covers at least 7 to 10 frames, containing at least: 5 brood frames, 2-3 frames of food, and 1-2 empty frames in order to allow colony growth.
5. The hives should be placed on site in the edge of the field 1-2 weeks before the start of the experiment (see schedule below) to allow the colony to adapt to the surroundings.

Shook swarm objective mode

1. With this method it is assumed that a pre -existing apiary will be modified for purpose. It is important to start at a time of year when the bees can draw out foundation into comb.
2. In the days leading up to set-up, queens in each colony are caged and returned to the colony to save time on set-up day. Colonies are removed from the apiary if they are expressing disease symptoms, significantly under-performing, or otherwise causing excessive between-colony variation. New colonies are imported if needed to reach the target colony number.
3. A number of empty hives equal to the target number of colonies is brought to the apiary, each stocked with brood chamber frames of new foundation, including honey supers with frames of foundation if the nectar flow warrants, and sugar syrup feeders. If affordable, it is good to start new colonies on factory-new woodenware to avoid any disease legacy effects.
4. Each hive in the apiary is moved aside and an empty hive set in its place. Roughly half of the frames of foundation are momentarily removed to create space, then the caged queen is suspended between two centre-most frames of foundation.
5. Combs of bees from the original colony are then sequentially removed and the adult bees shaken off the combs into the new box. Bees are bounced or brushed out of the supers and the bee-free combs returned to them and covered to discourage robbing behaviour.
6. Once all bees are shaken into the new hive, the frames of foundation initially removed are gently returned to the new boxes. Unless there is a strong nectar flow in progress, it is advisable to feed experimental colonies sugar syrup to encourage drawing out the new foundation.
7. The old bee-free boxes of combs are then removed from the experimental apiary and the combs used as needed elsewhere as supplemental brood or feed.
8. After one day, the caged queens in experimental colonies are released. Colonies are subsequently monitored for queen performance and normal colony development. Poor-performing queens are replaced as needed to minimize within-apiary experimental error. Once colonies reach a development state consistent with the experiment's objectives, treatments may be applied and the experiment started.
9. The expected outcome of this manoeuvre is a high degree of within-apiary consistency in colony developmental state.

Classical objective mode

1. The goal is field colonies equalized with regard to bees, brood, mites, and food resources.
2. Empty hives are pre-stocked with brood, empty combs, syrup feeders, and a caged queen in advance of receiving worker bees. Hive entrances are screened to temporarily trap bees:
   a. experimental colonies often need to be moved to a permanent site and away from the source colonies from which workers are collected
   b. a period of in-hive confinement helps bees orient to their new hive and queen.
3. Brood for incipient experimental colonies can be collected from the same source colonies used to collect adults. An equal quantity of brood is then assigned to experimental colonies without regard to source. We do not prescribe "random" brood assignment because a higher priority should be placed on equalizing quantity of brood over concerns of non-random assignment of brood. Efforts should be made to equalize the relative quantity of sealed versus open brood.
4. The investigator can also equalize the initial number of cells of honey or pollen or empty cells.
   It may be simpler to provide only brood or empty cells and to provide uniform nutrition across the experiment using sugar syrup and protein supplements.
5. Variation due to bee genetics is minimized by providing each colony a sister queen reared from the same mother and open-mated in the same vicinity.

6. Adult bees are collected for experimental set-up by shaking workers from a number of source colonies into one large, common, ventilated cage, allowing workers (and diseases and parasites) to freely mix. Sometimes, it helps minimize loss from flight to first spray bees on the comb with water mist. The cage is maintained in cool conditions to prevent bee death from over-heating for at least 24 hours to allow thorough admixing of bees, resulting in a uniformly heterogeneous mixture. The weight of bees collected (kg) should exceed the target weight of bees needed for the study by at least 2 kg, to account for bee loss through death or flight. Bee survival in the cage is greatly improved if the investigator designs it to accommodate 5-6 Langstroth sized brood combs to provide clustering surface.
7. In order to equalize initial colony populations, it is preferable to make colony-specific caged cohorts. Empty screened cages, ideally made to fit on top of an empty hive, are each pre- weighed or tared with a balance in the field. The large common cage is opened, the bees sprayed with water to reduce flight, then bees transferred from the common cage into the smaller colony-specific cages with the aid of cups or scoops. Bees are added or removed from each colony- cage until the target weight is achieved and recorded, preferably ≥ 2 kg.
8. A sample of ca. 300 workers is collected from each incipient colony into a pre-weighed or -tared screw-top container, weighed fresh, then the number of bees counted in the lab to derive a colony-specific measure of average fresh weight of individuals (mg per bee). To count bees it is necessary to first immobilize them, either by freezing them or non-sacrificially with CO2 narcosis. Dividing initial colony cohort size by average fresh weight of individuals (mg) gives initial bee population for the colony.
9. A variation of steps 7 and 8 is available if the investigator is using nucleus hives small enough to weigh in their entirety in the field. In these cases, the intermediate step of a colony-specific cage is not necessary and the investigator can scoop bees from the common cage directly into the pre-weighed or tared hive. The net weight (kg) of bees is recorded, then initial population determined the same way as given in step 8.
10. If initial measures reveal outliers in the amount of bees, brood, honey, pollen, and empty cells, corrective action should be taken. In general, corrections aimed at minimizing experimental error are permissible until the point at which treatments are begun.
11. When hives are moved to the experimental site over-heating is a risk, and hives must be kept as cool as possible. There is an advantage to setting up colonies late in the day and moving hives to the experimental site at night. Not only is it cooler, but once hives are unloaded and entrances opened, the bees do not fly because of the darkness and this protracted period inside the hive seems to help them orientate to the new queen and reduces drifting. Colonies can be given sugar syrup after they are unloaded or 24 hours later after bees have settled down.
12. Apiaries should be arranged to limit worker drift between colonies. This can be done by “complicating” the visual field of bees with orientating landmarks near their nest entrances. This can be as simple as using rocks or trees or more deliberate such as painting varying geometric shapes on hive fronts.

References

Delaplane et al. (2013) Standard methods for estimating strength parameters of *Apis mellifera* colonies. Journal of Apicultural Research Vol. 52 . DOI 10.3896/IBRA.1.52.1.03.

Human et al. (2013) Miscellaneous standard methods for *Apis mellifera* research, Journal of Apicultural Research, 52:4, 1-53, https://doi.org/10.3896/IBRA.1.52.4.10

Medrzycki et al. (2013) Standard methods for toxicology research in *Apis mellifera*. Journal of Apicultural Research 52(4) DOI10.3896/IBRA.1.52.4.14

WP1.2.2 Sourcing *Osmia* pupae and nests

S Hodge, R Dean, S Hagenbucher, M Albrecht

11.02.2019

Pupae

Osmia pupae can be obtained from suppliers such as Wildbiene & Partner, RedBeeHive, etc..
This will be in the range of around 100 pupae per trap nest, so around 300 pupae per site.

The providers of *Osmia* pupae will ensure as far as possible that the sex ratio per trap nest and site will be approximately 1:1 (females:males) by selecting a ratio of 1:1 large (presumably females) : small (presumably males) cocoons.

Pupae should best be stored at around 2°C. If this is not possible, they should be stored at a maximum average temperature of 4°C.

It is very important that temperature fluctuations are minimal, and that maximum temperatures do not exceed 5-6°C even for a short time.

To break diapause, move the pupae to 10°C for two to three weeks.
Around that time the first males will emerge (see also protocol WP1.2.4).

Trap nests

All partners will use standardized trap nests using cardboard tubes.
This will contain approximately 100 cardboard tubes.

The nests will be supplied assembled to each partner, along with brackets to fix the nests to a support pole (see protocol WP1.2.4).

The nests will be posted out from January; posting will be staggered so that countries with early flowering periods will receive the traps first.

WP1.2.3 Obtaining *Bombus* colonies

S Hodge, M Rundlof

08.03.2019

Colonies

- There will be three ***Bombus terrestris*** colonies at **each** of the WP1 field sites;
  each country will therefore need **48 colonies** in total. However, it is advisable to order **a couple of extra colonies** as backup since something can happened during transport (e.g. the queen dies).
- Mainland Europe sites will use subspecies ***Bombus terrestris terrestris***;
  the UK and Ireland will use subspecies ***B. terrestris audax***.
- Use a commercial supplier's Standard *Bombus terrestris* hives.
- These hives contain around **80 workers** and should last **6+ weeks**.
- These hives are supplied with a 1.4 L bottle of feed; this feed should be used when the bees are in storage, and removed once the bees are on site and flowering of the target crop has started.

Purchasing Bombus

- Contact the supplier to make sure they can provide the number and type of nests you require at the appropriate time (see WP1.2.4 for guidance on sentinel bee set up). It is advisable to **contact the supplier at least a month in advance** to inform about the needed number of colonies and the approximate date(s) of delivery. Let them know that the colonies will be used for research and that it is therefore extra **important that the colonies are equal in size** (brood, brood development and worker number) at delivery.
- When talking with the supplier and ordering the colonies, ask for the **cotton** to not be added to the colonies, but to be delivered in a separate box. This is to aid the confirmation of a natal queen and estimation of the number of workers before field placement (see WP1.2.4).
- If you are buying nests from outside of your own country, please check if there are any import or legislative requirements associated with this.

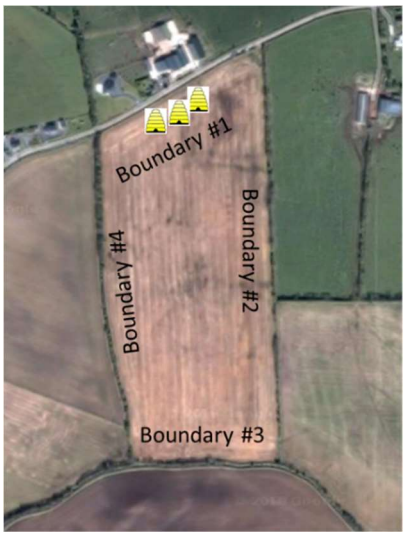
WP1.2.4 Hive, nest, colony installation on site

S Hodge, J Stout, R Dean, S Potts, E Attridge

12.03.2019

General

- At each site we will install
  - three *Apis* hives
  - three *Bombus* colonies
  - three *Osmia* trap nests
- The hives / nests will be placed along a linear field boundary (Boundary #1 see WP1.3.1)



(images from Google maps)

- Do **NOT** use the **north**-facing boundary (Boundary #3 above)
- The bees will be positioned at three stations along the **SAME** boundary: one station for *Apis* hives, one station for *Osmia* nests and one station for *Bombus* colonies
- For ease of set up, the boundary used should be accessible by foot, and preferably be close to a road. Remember the beekeepers will need to access the hives on a fairly regular basis.
- To avoid interference among the three species, the stations should be **at least 5 m apart** (but can be much further apart).
- The three *Apis* hives, *Osmia* nests and *Bombus* colonies should be given code numbers so that data or notes relating to specific entities can be related easily (see WP1.1.2).
- *Apis* hives should be labelled at each site as: A1, A2, A3
  Bombus colonies should be labelled at each site as: B1, B2, B3
  Osmia nests should be labelled at each site as: O1, O2, O3
- It is envisaged that the installation of the different species in a site could be staggered in time to account for differences among set ups and acquisition processes for *Apis*, *Bombus* and *Osmia*
- Set up date for each species at each site will be recorded as part of the basic site data (WP1.1.3)
- Overall, because of the short duration of flowering and amount of sampling that is required, all three species must be in position when flowering commences, from 3 to 7 days before the crop flowering starts.
- Make your best effort to set up of hives/nests/colonies so that in each site they are in situ at roughly the **same time relative to flowering for each site**. One way to do this is ask growers for estimated flowering start dates and deploy hives in the order expected by growers, but be prepared to be flexible if weather changes (e.g. cold spell or hot spell).
- Seek advice from local growers on the typical time of flowering of apples and oil seed rape in your country. Try and predict any time gradients in flowering that could occur due to location and aspect of the sites.
- Keep in regular close contact with growers as the flowering period approaches as they will have a good sense of when flowering is likely to start based on weather conditions etc.

Apis

- See **WP1.2.1** for further details of hive set up
- There will be **three** *Apis* hives placed out at each site
- Hives should be around **2 m** apart, and at least **5 m** from the *Osmia* nests and *Bombus* colonies.
- The hive entrances should be **south** facing.
- The main consideration should be to standardize the *Apis* colonies **WITHIN** each country.
  Aim for within-country uniformity in terms of boxes used, colony strength, genetic origin etc
  (e.g. each country should use local bees and local boxes).
- The colonies should be standardized as much as possible prior to being placed out in the field (see WP1.2.1)
- At the beginning of flowering we would expect hives to be of **typical strength** for the season and location. The initial colony strength assessment (WP1.5.1) will record this information.
- Bees should have 'typical' or acceptable low levels of pathogens and pests (see WP1.5)
- We would expect *Apis* hives will be on site approximately **1 week before** flowering of the target crops begins as this is typical beekeeping practice. Keep in regular close contact with growers as the flowering period approaches as they will have a good sense of when flowering is likely to start based on weather conditions etc.

Bombus

- There will be **three** Standard *Bombus terrestris* colonies at each site (see below).
- This will be *B. terrestris audax* for UK and Ireland; *B. terrestris terrestris* for all other countries.
- Details of how you should acquire your colonies are provided in **WP1.2.3**
- These nests come with a nutrition system in the form of a 1.4 L feed bottle. The bees should be fed this nutrient while they are in storage, but **removed at field placement** at start of the crop bloom (use the BBCH scales for the two focal crops to track crop phenology).
- Before field placement, identify the presence of a natal queen, count the number of workers per colony and note along with colony ID, and collect **4 workers** for pre-screening of pathogens (see WP1.4.4) and measurement of wings and intertegular distance (done by UMONS). Add a thin sheet of cotton on top of the brood and weigh the colony, excluding the outer cardboard box and the feed bottle (see WP1.5.9). All this can preferably be done in a dark room under red light (headlight with red light works okay).
- Locate a suitable spot in the field for placement of the bumblebee colonies. This should preferably be in the **shade** during the hot midday-afternoon sun and **sheltered** from wind by vegetation to the north.
- Colonies should also preferably be placed in a **protective structure** to prevent badger predation, ant attacks and sheltered from rain. This can be solved in many ways, from inexpensive plastic boxes (but ventilation is required and wood may be preferable), through modified honey bee hive boxes to specific houses (see pictures below for inspiration). This system should be standardized within each country but does not need to be consistent across the whole PoshBee network.
- The setting up of the *Bombus* nests could be staggered to represent the sequence of flowering commencement at your field sites. The *Bombus* nests will last around 6 weeks. We would expect these nests to be on site **a few days before** flowering of the target crops begins. Keep in regular close contact with growers as the flowering period approaches as they will have a good sense of when flowering is likely to start based on weather conditions etc.
- Therefore, consider the time of flowering of apples and oil seed rape in your country, and any time gradients in flowering due to location and aspect of the sites, to plan when nests should be **ordered** and then **moved on site**.
- Depending on whether the oil seed rape and apples flower at similar or very different times, it may be required to **order *Bombus* in two batches**.

Specific bumblebee houses and modified wooden honeybee hive boxes for housing the bumblebee colonies.

Osmia

- There will be **three** *Osmia bicornis* nests placed out at each site.
- These *Osmia* nests will be a standard design (RedBeeHive, see photos below).
- The central chambers will be loaded with *O. bicornis* pupae.
- Each nest will be initiated with 100 pupae. Try to balance the number of male and female ('small' and 'large') pupae over all of your sites.
- For details on how to obtain *Osmia* pupae and trap nests refer to **WP1.2.2.**
- The nests should be attached to a pole / poles 1-1.5 m off the ground (see similar set up below)
  Each country can decide whether they use three separate poles, or attach all three trap nests to a single pole, as long as the same procedure is followed at all sites in that country.
- The brackets needed to attach the nests to the poles will be provided with the nests
- Nests do not require a roof, although they may benefit from some form of shading in hot climates and some protection from extreme cold/ wet weather
- All nests should be facing the same direction: this will generally be **south-facing** for most countries.
- Nesting tubes should have their entrances tipped a few degrees (<10°) **down from horizontal** to reduce the risk of water accumulation during heavy rain
- Ideally the *Osmia* should be emerging, just as flowering is starting. Additionally, the *Osmia* must start emerging as the nests are being placed out in the field as we require them to be active during the short crop flowering period.
- However, care must also be taken to ensure the adults do not emerge too many days prior to the start of flowering, as the bees could abandon the nests if they have to travel considerable distances to forage.
- Synchronising the emergence of your *Osmia* with the start of flowering therefore requires some consideration.
- Consider the time of flowering of apples and oil seed rape in your country, and any time gradients in flowering due to location and aspect of the sites.
  - Keep in regular close contact with growers as the flowering period approaches; they will have a good sense of when flowering is likely to start based on weather conditions etc.
  - monitor weather and phenology, to anticipate bloom
  - use early flowering species such as *Prunus spinosa* (blackthorn/sloe) and cherries as an indication of how the season is progressing
- The bees will emerge slower in March than during May, although the emergence of adults can be managed to a slight extent by warming to accelerate emergence as required.
- The *Osmia* will be provided as pupae with fully developed adults inside
  The pupae should be stored at 4°C.
  It is advisable to incubate the pupae in the containers that will be used for their release.
- To break diapause, move the pupae to 10°C for two to three weeks. Around that time the first males will emerge. Keep in mind, that this process is also affected by length of hibernation.

- Check your bees daily for emergence. If they start to hatch early, you can always store at 0°C-4°C for one or two weeks to slow down the other bees.
- Maybe move 10 or so cocoons out of the fridge in March and observe how long they need to hatch. This will give you a feeling for your batch of pupae and their development rate.
  (use these bees to send to ANSES for pathogen pre-screening)
- After 10 days in the field, remove the emergence tube and replace with spare cardboard tubes. This is so any pests/parasitoids that were present in the founding stock do not emerge and immediately contaminate the new nest tubes, developing larvae and pupae.

The RedBeeHive Osmia bicornis nest

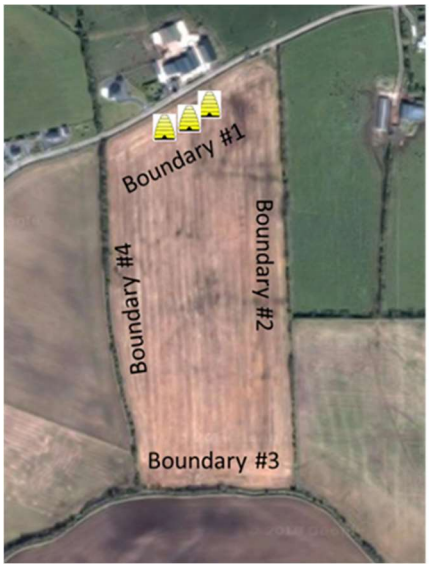
WP1.3.1 Basic Site Landscape Data

S Hodge, O Schweiger, S Potts

11.02.2019

General site data

1. To initiate the WP1 field work databases, we require general information for each site at the start of the field season (see WP1.1.4). We will also collect some basic landscape data early on in the project (possibly during the site selection phase).
2. We estimate this task will take **1 hour** per site
3. At each site, number the field boundaries clockwise **1-4.**
   Start at the boundary containing the sentinel bees, which is assigned **boundary #1.**
   (it is expected that in many cases Boundary #1 will be a south-facing boundary; **WP1.2.4**)
4. If the field is not square / rectangular, or has more than 4 boundaries, use the major boundaries and/ or number the major boundaries 1-4 as best you can.



(images from Google maps)

- At each site:

  i) each **field boundary** of the target crop will be classified into one of **six** categories.
  If there is more than one boundary category present use the dominant category.

  ii) the land adjacent to each boundary, will be classified into one of **thirteen land types**

  The codes (below) are based on the EUNIS system (see Appendix) that will be used in the more detailed Landscape Habitat classification work for WP1 (e.g. WP1.3.2).
  Add these data to the **Summary Table** for each site (see below).

- At this stage 'semi-natural' habitats will be pooled into one category.
  Please make a note of what your semi-natural habitat consists of and it will be allocated a higher level EUNIS code at a later date.

- In general, if you are unsure about a habitat category, make a note and it can be acted upon later.

Boundary Feature	**Code**
No boundary - fields run/ merge into each other	NB
Bare boundary (wire fence; wooden fence; stone wall)	BB
Hedgerow (mixed) / buffer zones / flower strips	FA
Windbreak or monoculture hedge	G5
Woodland edge	E5
Ditch / grassy verge	E2
Habitat	**Code**
Apples	APP
Oil seed rape	OSR
Pasture/Improved Grassland/	E2
Coniferous woodland	G3
Deciduous woodland	G1
Mixed woodland	G4
Horticulture other than apples	I1.2
Cereals/ arable crops other than OSR	I1.1
Bare tilled arable land	I1.5
Semi-natural habitat/Meadows	SN
Parkland	I2
High density housing / urban	J1
Low density housing / suburban	J2

EUNIS Habitat codes. (https://www.eea.europa.eu/data-and-maps/data/eunis-habitat-classification)

Habitat level	EUNIS habitat code	EUNIS habitat name
1	C	Inland surface waters
1	D	Mires, bogs and fens
1	E	Grasslands and lands dominated by forbs, mosses or lichens
1	F	Heathland, scrub and tundra
1	G	Woodland, forest and other wooded land
1	H	Inland unvegetated or sparsely vegetated habitats
1	I	Regularly or recently cultivated agricultural, horticultural and domestic habitats
1	J	Constructed, industrial and other artificial habitats
2	E1	Dry grasslands
2	E2	Mesic grasslands
2	E3	Seasonally wet and wet grasslands
2	E4	Alpine and subalpine grasslands
2	E5	Woodland fringes and clearings and tall forb stands
2	E6	Inland salt steppes
2	E7	Sparsely wooded grasslands
2	F1	Tundra
2	F2	Arctic, alpine and subalpine scrub
2	F3	Temperate and mediterranean-montane scrub
2	F4	Temperate shrub heathland
2	F5	Maquis, arborescent matorral and thermo-Mediterranean brushes
2	F6	Garrigue
2	F7	Spiny Mediterranean heaths (phrygana, hedgehog-heaths and related coastal cliff vegetation)
2	F8	Thermo-Atlantic xerophytic scrub
2	F9	Riverine and fen scrubs
2	FA	Hedgerows
2	FB	Shrub plantations
2	G1	Broadleaved deciduous woodland
2	G2	Broadleaved evergreen woodland
2	G3	Coniferous woodland
2	G4	Mixed deciduous and coniferous woodland
2	G5	Lines of trees, small anthropogenic woodlands, recently felled woodland, early-stage woodland, coppice
2	I1	Arable land and market gardens
2	I2	Cultivated areas of gardens and parks
2	J1	Buildings of cities, towns and villages
2	J2	Low density buildings
2	J3	Extractive industrial sites
2	J4	Transport networks and other constructed hard-surfaced areas
2	J5	Highly artificial man-made waters and associated structures
2	J6	Waste deposits

Basic Landscape Data Sheet

Add habitat codes for **B**oundaries (B1-B4) and **A**djacent **F**ields (AF1-AF4) for each site.

Number boundaries from 1-4, clockwise, starting at the boundary containing the beehives.

Country: _____

Date	Surveyors	Site code	B1	B2	B3	B4		AF1	AF2	AF3	AF4	Comments
		OSR_1										
		OSR_2										
		OSR_3										
		OSR_4										
		OSR_5										
		OSR_6										
		OSR_7										
		OSR_8										
		APP_9										
		APP_10										
		APP_11										
		APP_12										
		APP_13										
		APP_14										
		APP_15										
		APP_16										

WP1.3.2 Complex Landscape Data

S Hodge, J Stout, O Schweiger

11.02.2019

1. Various landscape measures will be collected based on the WP1 site network using several GIS data systems.
2. As part of **WP1.3.1** and the **selection of field sites**, WP1 partners will send site **GPS coordinates** to **UFZ** coordinators and they will extract some indications of agricultural and semi-natural area within a radius of 3-5km. This is to help obtain a suitable gradient of agricultural intensity / semi-natural areas.
3. A **more detailed landscape characterisation** of the habitat in a 1 km radius circle around the target sites will also be performed by **UFZ**. The intention is to classify all habitat patches with a much higher spatial resolution compared to the approach mentioned in **(2)** above.
4. **UFZ** will generate **detailed GIS maps** based on polygons of habitat patches generated from digitalisation of satellite maps and other data sources.
5. EUNIS codes will be assigned to each polygon.
   This preliminary assignment will be assessed by WP1 field workers who will test the accuracy of these maps by **ground truthing**. To avoid interfering with the intensive sampling season, this ground truthing could be performed after the sampling season has been completed. Details of this task will be provided by UFZ at a later date.
6. Additionally, as part of **WP1 Task 1.4**, TCD will collect land use data on **Ireland** and **Italy** at a national scale via the Land-Parcel Information System (LPIS) and other national data sources. These data will feed into the MUST-B bee health model lead by Aarhus University. These data will **NOT** be collected for other WP1 partners.
7. In summary, WP1 partners:
   - are **NOT** required to collect any landscape data via GIS or satellite imagery
   - **should** provide site GPS coordinates to UFZ for data to be extracted remotely
   - **are** required to do the basic landscape classification outlined in protocol WP1.3.1
   - **will be** required to perform some ground truthing of habitat types for UFZ late in the field season

WP1.3.3 Floral survey of target field boundaries

S Hodge, J Stout, S Potts

06.03.2019

1. The aim of this survey is to collect basic data that describes the floral abundance and diversity in the immediate area of each site. The survey is also used to provide examples of the most common flowering plants to build a pollen library to aid identification of pollen in beebread (protocol 1.4.5).
2. The floral survey will be performed only **once** at each site
3. The floral survey should be performed in the **middle** of the flowering period for each crop within each country. Surveys can be carried out in cooler or wetter weather when other active bee sampling protocols cannot be undertaken; but note if the weather is very cold or hard rain then some flowers may close.
4. At each site, the field boundaries should be numbered clockwise (**1-4**) starting at the boundary containing the sentinel bees, which is assigned **boundary #1. (see also WP1.3.1)**
   If the field is not square / rectangular, number the boundaries 1-4 in a sensible way.
5. Data on floral abundance and richness will be obtained by **quadrat sampling** the **field margin** (or buffer zone, field boundary etc).
6. Within each country, the **same person/team** should perform all the site floral surveys.
7. On each field margin **three areas** will be assessed. Sample areas that are **typical of the boundary habitat** in general (do not try and maximise the number of flowers present). To space out these areas approximately equally, divide the field margin into four, and perform a quadrat sample approximately **¼, ½** and **¾** along its length.
8. Because in some countries fields can be very large (> 1 km in length) record the **GPS** coordinates of each sampling point. Add these data to the site **Summary Table**.
9. In this survey we will count **floral units,** defined as '…(groups of) flowers that a small bee would have to fly rather than walk between'…(see below)].
10. The floral units we will count are those relevant to our study, and therefore only refer to flowers that would typically be pollinated by insects i.e. non-graminoid angiosperm flowering plants.

At each sampling zone along the **boundary**, we will:

a) assess flowers on the **ground**
b) assess flowers in the boundary **hedge** (or other boundary vertical structure) if one exists
c) collect samples of the assessed flowering plants to send off for pollen analysis (step 13)

1. We want to examine:

- **average species richness** per quadrat
- **total species richness** per site

Therefore, plants should be identified to **species level** where this is possible**.**
If a 'morpho-species' or 'Recognizable Taxonomic Unit' (RTUs) approach is used (or partially used), then species codes **must be consistent** across all sites, which is much easier if a single person carries out all floral surveys at all sites.

2. For each **1m²** quadrat on the **ground**, use the data sheet to record:

i) the **presence / absence** of each **species** that are **in flower**

ii**) score** each quadrat in terms of **total** 'floral unit' abundance':

Total Floral Units	Score
0	0
<10	1
10-100	2
>100	3

Place the quadrat in area of typical and representative flowering. If the floral strip or boundary is less than 1 m wide then include as much as possible into the 1 m quadrat, but **do not** make up 1 m^2 by taking multiple samples.

3. If there is a **hedge** present, place a **1 m²** vertical quadrat alongside the side of the hedge adjacent to the target field, at a height that reflects what is typical of that structure. For each **1 m²** quadrat on the **hedge**, use the data sheet to record all the flowers you see to a depth of approximately 30 cm:

i) the presence / absence of each **species** that are **in flower**

ii**)** score each quadrat in terms of **total** 'floral unit' abundance':

Total Floral Units	Score
0	0
<10	1
10-100	2
>100	3

If the hedge is less than 1 m high then include as much as possible into the 1 m vertical quadrat, but **do not** make up 1 m^2 by taking multiple samples.

If there is **no hedge** present, then 'vertical' floral richness and abundance will be given **default scores of zero**

4. In each country, for each crop (apple and OSR) the site with **the highest natural habitat level** should be chosen to collect flower samples to be sent to CREA to make reference slides to support pollen identification in beebread / pollen stores. In total 1 apple site and 1 OSR site.

Be careful not to collect protected species!

In each chosen site, on the central sampling zone of each of the 4 boundaries, both on the ground and in the boundary hedge, try to collect **6 floral units for each present species**.

If not possible to collect 6 floral units inside the quadrat, collect from near the quadrat if that species is present.

If the species is present in all boundaries collect only until 6 floral units have been reached.

Insert the floral units in paper bags (a traditional envelope size 11 x 23 cm, or smaller, is suitable but avoid bigger sizes), **one paper bag per species**.
Label the paper bag with the site code and name of the (morpho-)species.
If writing in the field use a graphite pencil to avoid risk of smudging. From each site same species samples can be grouped to obtain one sample per species per site, if identification is certain, otherwise keep samples separate.

When returning from the field **store the samples in paper bags at -20°C**.

Send to CREA together with bee samples for pesticide analyses (see Protocol 1.4.4 for address details).

To summarise, the following number of flower sampling events should take place in each country: 2 crops x 1 site x 4 boundaries x 2 heights = 16

The total number of samples collected will depend on the number of species present in the sites.

Researchers are free to collect additional samples of flowers of common species from other sites if they are not present at the two main sampling sites

5. We estimate this task will take **1 person 2-3 hours per site.**

Times will obviously vary depending on country, geography, the floral diversity of the field margins and presence /absence of any hedge structure.

To shorten the procedure in the field, plant species can be given code numbers in the field, and samples / photographs taken so that identifications can be made later back at home institute.

6. Kit list

The kit list should include: quadrat, tape measure, datasheets, plant ID guide, sample bags, pens/pencils, notebook, camera (to check species ID after field visit if needed).

A plant identification app for smart phones (eg: iplant, PlantSnap) may also be useful.

Floral unit clarification

For simplicity we will count floral units (flower heads, inflorescences etc) rather than individual inflorescences.

A floral unit can be defined differently for different species.

Flowers may be solitary, or they may be grouped together in an inflorescence (a cluster of flowers). An inflorescence has one main stalk, or **peduncle**. It may also bear numerous smaller stalks called **pedicels**, each with a flower at its tip. The arrangement of pedicels on a peduncle characterizes different **kinds of inflorescences**.

Please note, it is important that you don't under sell your floral units. The main example of this is flowers like daisies and other composites. These are often counted as a single flower, but they are a cluster of tiny flowers. Importantly, **not all of these flowers are open at the same time**.

Definitions and examples of different types of floral unit can be found at this website http://www.flowers-gardens.net/gardens/types-of-inflorescence.html which may help:

Summary table

Site:			
Date:			
Surveyors:			
Boundary	**Quadrat #**	**Latitude**	**Longitude**
#1	**Q1**		
(with hives)	**Q2**		
	Q3		
#2	**Q1**		
	Q2		
	Q3		
#3	**Q1**		
	Q2		
	Q3		
#4	**Q1**		
	Q2		
	Q3		

Record table - Ground Flowers

Site				Date								
	Boundary #1			Boundary #2			Boundary #3			Boundary #4		
Species	Q1	Q2	Q3	Q1	Q2	Q3	Q1	Q2	Q3	Q1	Q2	Q3
1.												
2.												
3.												
4.												
5.												
6.												
7.												
8.												
9.												
10.												
11.												
12.												
13.												
14.												
15.												
Quadrat #Species												
Quadrat #Units												
Boundary #Species												
Boundary # Units												
Site #Species												
Site # Units												

Record table - Hedge Flowers

Site				Date								
	Boundary #1			Boundary #2			Boundary #3			Boundary #4		
Species	Q1	Q2	Q3	Q1	Q2	Q3	Q1	Q2	Q3	Q1	Q2	Q3
1.												
2.												
3.												
4.												
5.												
6.												
7.												
8.												
9.												
10.												
11.												
12.												
13.												
14.												
15.												
Quadrat #Species												
Quadrat #Units												
Boundary #Species												
Boundary # Units												
Site #Species												
Site # Units												

WP1.3.4 Surveys of wild and managed pollinator insects

S Hodge, J Stout, S Potts

05.03.2019

1. The aim of this process is not to provide a detailed inventory of the species occurring at each site, but to gain information on the abundance and diversity of pollinators as an aid to describing each site in terms of general pollinator 'health'.
2. Individual partners, or groups of WP1 partners, may extend the research on pollinators if they wish, as long as care is taken not to influence the results of WP1 e.g. no destructive sampling should occur, especially when the sentinel bees are in position; excess specimens should not be taken for identification; no procedures should be performed that may influence the behaviour or health of the sentinel bees [e.g. baiting, providing extra feed resources, clearing vegetation etc]
3. Counts will be made of pollinators assigned to broad categories (see Appendix 2):
   honey bees (*Apis*);
   bumble bees (*Bombus*);
   solitary bees;
   hoverflies (Syrphidae);
   butterflies (Lepidoptera).
4. Data on pollinators occurring at each site will be obtained by **timed transect sampling**
   (based on methods given by Westphal et al. 2008; Stanley & Stout 2013).
5. When sampling, the recorder should walk at a slow, steady pace and record the insects observed during a **5- minute** period, along an approximately **50m long**, **2m wide** strip.
6. Record **all** the pollinator insects you see, including those that are visiting flowers, those engaged in pollinating activities, or those just flying around.
7. Transect counts should only be performed **warm days with little wind**.
   Transects should be performed between **10am and 4pm**.
   The **time of day** when the survey is performed should be recorded.
8. If possible, try to organize sampling so the same site is **not** always sampled at the same time of day.
9. Record **temperature 1 m** above the ground level in shade using a **thermometer**.
   Estimate **cloud cover** (%)
   Estimate **wind speed** using Beaufort scale (see Appendix 1).
   Add these results to the table provided.
10. Transect counts will be performed on **three** occasions at each site:
    i) near the **beginning** of the crop flowering period
    ii) **during** the middle of the crop flowering period;
    iii) towards the **end** of the crop flowering period.
11. Try to perform each round of surveys in the same crop type in a short a time as possible.
    [The timing of the three survey rounds for apple and OSR will very probably differ]
12. On each occasion, counts of pollinators will be performed in **four** locations at each site:
    i) **two** within the target crop
    ii) **two** along a field boundary
13. For the within crop samples try and sample close to the centre of the crop where possible.
    If fields/orchards are large, and the centre is far away, sample at least 30 m from the edge of the crop.
    The two samples should be at least 30 m apart.
    The transect can consist of a strip 1m either side of the recorder's transect path
14. For the boundary samples, the boundary with the **sentinel** colonies should **never** be used.
    Number the boundaries clockwise; with the **sentinel bee boundary assigned #1**.
    This will give the boundaries the same numbering scheme as for the floral surveys (**WP1.3.3**)
    The actual field boundary should be sampled, **NOT** the edge of the crop.
    [This will help to investigate relationships between wild pollinator diversity and floral diversity in the field boundaries, and in the descriptions of the sites in terms of floral/ pollinator 'health']
15. The transect will consist of a 2 m wide strip from the field edge/ buffer zone.
    Include pollinators on any edge vegetation within the 2 m strip up to a height of 2 m
    (eg hedgerow shrubs, small trees)
16. The **same person/team** should carry out all surveys at all sites and on both crop types.

17. Try to avoid 'double counting' the same specimens.
18. Use pre-prepared data sheets to record taxa as the survey progresses.
19. We estimate that this protocol will take **30 person minutes per site**
    (times will vary due to geographic location; floral diversity of the field margins etc).

Appendix 1.

Beaufort Wind scale

FORCE	**EQUIVALENT SPEED**		**DESCRIPTION**	**SPECIFICATIONS FOR USE ON LAND**
	miles/hour	knots		
0	0-1	0-1	Calm	Calm; smoke rises vertically.
1	1-3	1-3	Light air	Direction of wind shown by smoke drift, but not by wind vanes.
2	4-7	4-6	Light Breeze	Wind felt on face; leaves rustle; ordinary vanes moved by wind.
3	8-12	7-10	Gentle Breeze	
4	13-18	11-16	Moderate Breeze	Raises dust and loose paper; small branches are moved.
5	19-24	17-21	Fresh Breeze	Small trees in leaf begin to sway; crested wavelets form on inland waters.
DO NOT SAMPLE ABOVE THIS LEVEL OF WIND				
6	25-31	22-27	Strong Breeze	Large branches in motion; whistling heard in telegraph wires; umbrellas used with difficulty
7	32-38	28-33	Near Gale	Whole trees in motion; inconvenience felt when walking against the wind.
8	39-46	34-40	Gale	Breaks twigs off trees; generally impedes progress.
9	47-54	41-47	Severe Gale	Slight structural damage occurs (chimney-pots and slates removed).
10	55-63	48-55	Storm	Seldom experienced inland; trees uprooted; considerable structural damage occurs.
11	64-72	56-63	Violent Storm	Very rarely experienced; accompanied by wide-spread damage.
12	73-83	64-71	Hurricane	--

Pollinator survey results/ tally sheet

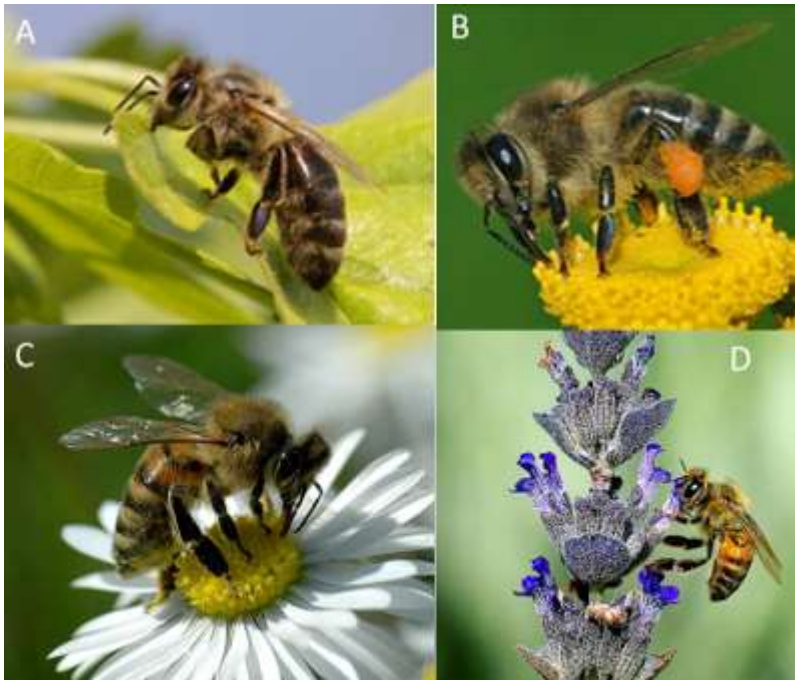
Site:	**Date:**		**Surveyor(s):**	
Time:	**Temperature (°C):**		**Cloud cover (%):**	
	Beaufort scale:			
	Counts of individuals			
	Boundary # ___	**Boundary #___**	**Field #1**	**Field #2**
Apis				
Bombus				
Solitary bees				
Syrphidae				
Lepidoptera				

Pollinator survey results/ tally sheet

Site:	**Date:**		**Surveyor(s):**	
Time:	**Temperature (°C):**		**Cloud cover (%):**	
	Beaufort scale:			
	Counts of individuals			
	Boundary # ___	**Boundary #___**	**Field #1**	**Field #2**
Apis				
Bombus				
Solitary bees				
Syrphidae				
Lepidoptera				

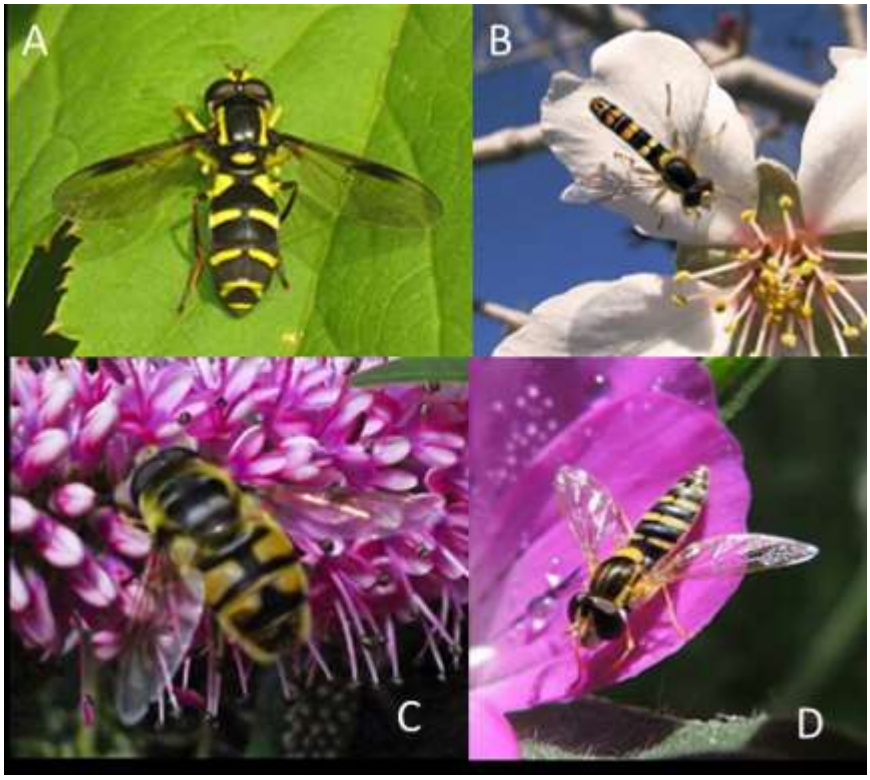
Appendix 2 Identification guide

Honey bees



Bumble bees

Syrphidae



Solitary bees

Lepidoptera

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Honey bees: A. gbohne. B. Wikipedia. C. Riccardo Cuppini. D. Danny Perez Photography

Syrphidae: A. Martin Cooper. B. Jacinta lluch Valero. C. Mick Talbot. D. Orangeaurochs

Leipdoptera: A. Dan Davison. B. Charlene N Simmons. C. Michael Mueller. D. Amanda Slater

Bumble bees: A. S. Rae. B. S. Rae. C. S. Rae. D. Thomas Bresson

Solitary bees: A. Jürgen Mangelsdorf. B. Bramblejungle. C. Jürgen Mangelsdorf. D. S.Rae

WP1.3.5 Grower Survey

T Breeze

07.03.19

Objectives The survey is designed to collect detailed information on what pest controls are likely to be used in the fields and orchards where the PoshBee study is taking place. This will give us essential background data for comparison with the chemicals identified by the analysis performed for WP2. If the data collected in this survey are useful for your own PhD work or other research papers, you may use them but, due to GDPR restrictions, you must consult Tom Breeze first.

The survey should take about 20-30 minutes to complete depending on how complete each farmers records are. You will need to note down which site you are collecting the data from using the PoshBee site code.

Timing

1. At the start of the field season you should tell farmers that they will be asked a few questions at the end of the field season about their pest control measures this year.
2. Early in the field season, the survey should be translated into your local language by a member of the project team in your country (see below for guidelines)
3. About 2 weeks before the field work is completed, send the translated survey to the farmers and make sure they have received it and encourage them to look over it.
4. Once the fieldwork is completed, arrange a time with the farmer to conduct the survey.
5. Conduct the survey, entering the respondent's answers into the online survey (links). At the end of the survey you must inform farmers that you will call again to collect basic information on the yields of their crops.
6. At harvest, return to the farmers to collect the information on yield. These questions are not in the online survey but will be provided separately.

Conducting the survey (general)

1. Towards the end of the field season, you should agree with the farmer on when and how the survey is to be conducted.
    - It is strongly recommended that you read the questions to the farmer either over the phone or face to face and fill in their responses using the online survey (links) on a laptop or tablet.
    - If they are unwilling or unable to do the survey over the phone or face to face, you can send them a link to the online survey (there is a drop down menu that allows them to select the appropriate language.
    - Paper versions are also an option, however this should only be done if a farmer insists as paper versions are very likely to be lost and will need to be produced especially.
    - If you collect the data in a face to face or phone interview, please enter the results in English into the online survey (hosted on Qualtrics) directly, either as you are conducting the survey or immediately after from paper/typed notes. This is to avoid complications from people having to read each other's handwriting and to ensure that the responses are standardised.
2. Ethical considerations
    - If you collect the data in a face to face or phone interview, you must read the ethics statement to the respondent at the start of the survey to be GDPR compliant.
    - Please stress that the data collected are only available to yourselves, myself and a few members of the WP1/WP2 management teams (full list available on request), otherwise only summary data will be printed.
    - Although no personal identifiers are published and the survey does not ask for any personal details, because the survey has some questions with open answers, which could be used for participants to identify themselves in some way or where answers could be analysed to reveal personal information, the survey is not legally considered to be anonymous, so please do not claim that it is.
    - GDPR rules prevent the sharing of this data with anyone outside of the project.
3. In the question asking for the farm name, please enter the PoshBee site code.

4. When conducting the questionnaire, you are allowed to give example answers either on request or to prompt a response.
5. If you are using a paper questionnaire, some of the questions are marked in blue. These questions only need to be asked if respondents answer previous questions in a certain way (e.g. asking how long the field has been under organic management only if they have indicated that they are certified as organic). If you are using the online survey they will only display if the relevant answers are selected.
6. Q8 asks farmers for information on chemical applications.
    - Product name should be the name on the label not the active ingredient
    - There are 3 slots for date and rate of application. For each date, please include a separate rate of application as farmers may apply more or less at different times.
    - If you need more than 3 dates, please make a note of any extra dates and application rates and e-mail this to Tom Breeze. We have not added more than 3 dates to avoid the survey being hard to read.
7. When collecting answers for Q5 and Q8, please be as detailed as possible: it's better to have too much information and cut it down than to have too little and have to go back.

Translations

1. If you are responsible for translating the survey into your local language, please let Tom Breeze know. Tom will then send you the word copy of the questions. Tom will then add your translation to Qualtrics' translation feature.
2. Please put the translated text below the English original in the word document. For tables, please use the extra blank row and column provided.
3. You must keep the questions in the same order and format as the original English version.
4. Please translate the text as close as possible to the original wording and do not add subjective or colloquial terms. In particular: the questions must convey that we are talking about the study field and this year only, not other years or other, local fields.
5. Let Tom know immediately if there are any questions that are difficult or impossible to properly translate and we will try to arrange an alternate wording.
6. It is planned to have all translations completed by 20/05/2019.
   Please contact Tom Breeze if you encounter problems with this completion date.
7. Once Tom has uploaded it, you will be asked to look through the survey and sign off on it before it goes live. Word copies will then be made available.

WP1.4.1 Water from puddles

S Hodge, J Stout

11.02.2019

It was originally planned to collect samples of ground water from puddles at the target sites and analyse this water for chemical residues. This was because honeybees often drink from puddles, and *Osmia* use mud to seal their nests, and this surface water therefore represented another route via which the sentinel insects could come into contact with agrochemicals.

However, this was dropped prior to the submission of the PoshBee proposal, but due to an editing error was left in the Grant Agreement. No resources were allocated for the chemical analysis required for agrichemical detection.

Therefore the sampling of water from puddles **WILL NOT BE PERFORMED**.

WP1.4.2 Collecting pollen from target crop and from foraging bees

S Hodge, M Laurent, J Stout, M Rundlöf

05.03.2019

Target Crop

1. **Pollen** will be collected from the target crop at each site to assess levels of **chemical** contamination
2. Be careful to change gloves and other necessary materials between sites and between collection of different substances (pollen from flowers, from *Bombus* legs or from *Apis* traps) to avoid cross-contaminations.
3. **Dried 'pure' pollen** will be obtained following the method of Botias et al. (2015)
4. Pollen will be collected from **1000 flowers** of the **target crop** at each field site.
5. Collect the flowers in the **middle** of the **flowering** period and place in clean **paper bags**.
6. Identify the bags with **COUNTRY CODE, CROP CODE, SITE CODE, SUBSTANCE CODE and SAMPLING DATE** as detailed in the site labelling scheme (WP1.1.2). For example, the label "ESP_APP_12_FP_19-04-2019" refers to a sample of flower pollen located on the site **12** of **apple tree** orchard in **Spain** and collected on **April 19.**
7. Store the flowers in **coolers** in the field.
8. When possible, transfer the flower samples to **freezers** at -20°C until further handling.
9. To begin the pollen extraction, defrost flowers at room temperature.
10. Dry the flowers in an incubator or oven at 37 °C for 24 hours to facilitate pollen release from the anthers.
11. After drying, brush the flowers using a fine paint brush over food strainers to separate pollen from anthers and other debris
12. Sift the pollen through sieves of decreasing pore size (for example, pore sizes from 250 to 45 µm; oilseed rape pollen grain: Ø = 21-28 µm).
13. It is estimated that from c. 1,000 flowers you will obtain around 200-300 mg of dry pollen.
14. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date and weight of the sample of dry pollen.
15. Store the pollen in an Eppendorf tube or a polypropylene jar (depending on the collected quantity) in a freezer until sending to the analytical lab. Identify the Eppendorf with labels indicating **COUNTRY CODE, CROP CODE, SITE CODE, SUBSTANCE CODE** and **SAMPLING DATE** as described in the **WP1.1.2 Site labelling scheme**. (e.g. "IRL_APP_13_FP_19-04-2019")
16. Labels will be printed with a laser printer (to avoid smudging with humidity) and not hand written. Wrap the label around the Eppendorf.
17. The samples should remain in the freezer at -20°C until posted to NEBIH.
18. Pollen will be sent to NEBIH.

Pollen from foraging *Apis*

1. We will also collect **pollen** from foraging *Apis* returning to the hive. This is because some chemical analysis requires up to **5 g** of pollen; an amount which cannot be provided by the method above.
2. Use pollen traps that fit over the entrances of your *Apis* hives. There are many pollen traps available: use a version which is typical of those used by beekeepers in your country (see below)

3. Please inform your beekeepers that you plan to collect pollen that day.
4. Do not leave the pollen traps in place for too long a time as this will deprive the hive of nutrition; possibly 1 hour collecting over the three hives will suffice to collect the weight required.
5. Collecting should be performed late-morning to afternoon on a day with fine weather, so that worker bee foraging is occurring with high intensity.
6. Be careful to change gloves and other necessary materials between sites and between collection of different substances (pollen from flowers, from *Bombus* legs or from *Apis* traps) to avoid cross- contaminations.
7. Pool the pollen from the different hives at each site: one sample from pollen traps per site.
8. Store in polyethylene bags, clearly labelled with **COUNTRY CODE, CROP CODE, SITE CODE, SUBSTANCE CODE** and **SAMPLING DATE** as described in the **WP1.1.2 Site labelling scheme**. (e.g. "IRL_APP_13_AP_14-04-2019").
9. Use pre-labelled bags or pre-prepared printed labels if available.
10. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date, collectors and weight of pollen.
11. The samples should be stored in a freezer at -20°C as soon as this is possible and remain there until posted to NEBIH.

Pollen from foraging *Bombus*

1. Pollen from foraging bumblebees will be collected from the specimens sampled during the crop flowering period for nectar and pesticide residue analyses (WP1.4.3 and WP1.4.4). This is an insurance in case there is insufficient pollen stored in the colonies at the end of the study period.
2. **30 *Bombus*** will be collected as part of WP1.4.3 nectar+bee specimens for chemical residue analysis. These specimens will already have been frozen as part of the above protocols, and the specimens should be in storage in a freezer.
3. Be careful to change gloves and other necessary materials between sites and between collection of different substances (pollen from flowers, from *Bombus* legs or from *Apis* traps) to avoid cross-contaminations.
4. When removing the pollen from the legs of these bees, first prepare **1.5 ml Eppendorf tubes** for each site, clearly labelled with **COUNTRY CODE, CROP CODE SITE CODE, SUBSTANCE CODE** and **SAMPLING DATE** as described in the **WP1.1.2 Site labelling scheme**. (e.g. "IRL_APP_13_BP_05-07-2019").
5. Labels should be printed with a laser printer (to avoid smudging with humidity) and not hand written.
6. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date, number of bees processed.
7. Bring the dead bees from **one** site out of the freezer.
   Let the bees **thaw slightly**, but **minimize the time outside** the freezer as much as possible.
8. **Process the bees from each site separately**. Cover the work surface with **clean paper cover**, which should be replaced between each site's bees. Use soft and/or hard tweezers to remove the pollen from the bumblebee's legs and place the pollen in the pre-weighed and labelled Eppendorf tubes. **Clean the tweezers** between each site's bees using paper and 70% ethanol.
9. Weigh the total pollen sample and add this to the data summary sheet.
10. Put the bees back in the freezer as soon as possible.
    Place the pollen in a freezer at or below **-20°C** as soon as this is possible until it is posted.
11. The samples will be sent to PIWET.

References

Botias, C. et al. (2015.) Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. Environmental Science and Technology 49: 12731-12740

Postage

1. The samples will be shipped from one institution to another with **dry ice**, taking care to maintain the deep freeze chain (UN 1845 procedure requirements) (packed in cold resistant plastic containers or small plastic bags (double) surrounded by dry ice and packed into cardboard boxes). Samples will be posted with special care to avoid crushing (use rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements for dry ice shipments by air http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1 .
2. Packages should be sent at the beginning of the week (before Wednesday) to avoid delay and blockage during the weekend.
3. To reduce shipment costs as much as possible, each partner is recommended to organise the shipment when **ALL** of the samples are collected.

Summary of pollen collections from target crop

Site code	Date flowers collected	Time	Collector(s)	Date pollen extracted	Person extracting	Weight dry pollen (mg)
OSR_1						
OSR_2						
OSR_3						
OSR_4						
OSR_5						
OSR_6						
OSR_7						
OSR_8						
APP_9						
APP_10						
APP_11						
APP_12						
APP_13						
APP_14						
APP_15						
APP_16						

Summary of pollen collections from *Apis* pollen traps

Site code	Date	Time	Collector(s)	Fresh Weight (g)
OSR_1				
OSR_2				
OSR_3				
OSR_4				
OSR_5				
OSR_6				
OSR_7				
OSR_8				
APP_9				
APP_10				
APP_11				
APP_12				
APP_13				
APP_14				
APP_15				
APP_16				

Summary of pollen collections from *Bombus* legs

Site code	Date	Time	Person processing the sample	No.of Bombus	Weight empty tube (mg)	Weight filled tube (mg)	Weight Pollen (mg)
OSR_1							
OSR_2							
OSR_3							
OSR_4							
OSR_5							
OSR_6							
OSR_7							
OSR_8							
APP_9							
APP_10							
APP_11							
APP_12							
APP_13							
APP_14							
APP_15							
APP_16							

WP1.4.3 Collecting nectar from stomachs of *Apis* and *Bombus* for WP2

M-P Chauzat, A-C Martel, M-P Rivière, M Laurent, N Cougoule, D Michez, S Hodge, M Rundlöf
05.03.2019

General procedure

1. The aim of this process is to sample **nectar** from the stomachs of adult *Apis* and *Bombus*.
   The nectar will be used in the analysis of **agrichemicals** that form part of WP2.
   The bodies will be retained and sent to CREA for **pesticide residue** analyses.
   This will create a set of paired samples (nectar/bodies) that will tested for agrichemicals (WP1.4.4).
2. At each site, adult insects of **TWO** bee species (*Apis mellifera* and *Bombus terrestris*) will be collected **ALIVE**. Details for collecting method and quantity of bees for each species are given below.
3. In this document, **hives** refer to *A. mellifera* and **colonies** refer to *B. terrestris.*
4. At each site, collect samples of bees from all **THREE** hives and **THREE** colonies. The nectar from the bees of each species will be **pooled** to give **one sample** of nectar from **each species** for **each site**.
5. The same approximate amount of bees should be collected from each honeybee hive. The differences in quantities sampled between hives should be of max 30%. This is also the aim for bumblebee colonies, but here it is acceptable to collect the first 30 individuals that arrive to the colonies independent of home colony. If one hive or colony shows significantly lower flight activity, it should be noted. In this case, the samples from the two other hives/colonies should be higher to reach the total quantity requested.
6. The sampling will be performed only **ONCE** at each site. So that species can be compared directly, try to collect nectar from **the two species** at the **same site** on the **same day**.
7. It is estimated that this process will take one person approximately **1 hour per species**.
8. The sampling can be performed **during** the **middle** (preferentially) to **end** of the crop flowering period. Try to collect all the nectar from the bees on the same crop within a small time-window (3-4 days). This will mean visiting 2 or 3 sites each day.
9. Collect foraging bees returning to the hives / colonies and store them in labelled polypropylene **JARS.**
10. Bees can be put into torpor and easier to handle by placing them in a chilled box but it is important to proceed to the nectar collection as soon as possible.
11. If you have the necessary kit, the bees can be put to sleep using CO_2. In case of use of CO_2 kits (see Figure 1) and not a bottle of CO_2 (see Figure 2), use clean CO_2 mite shakers between each species and each site in order to avoid pesticide residue contamination. You should have enough CO_2 refills to anaesthetize all the bees.
12. Be careful to change gloves, collection jars and other necessary materials between bee species on the same site, and between sites to avoid cross-contaminations.
13. Be careful to identify the jars (on the cap and the container) with **COUNTRY CODE, CROP CODE**, **SITE CODE, SPECIES CODE, HIVE CODE, ANALYSIS CODE and SAMPLING DATE** as detailed in the site labelling scheme (WP1.1.2). For example, the label "ESP_APP_12_B1_NTOX_19-04-2019" refers to ***B****ombus* 1 colony, located on the site **12** of **apple tree** orchard in **Spain** collected the 19/04/2019 for nectar collection and pesticide residue analyses.
14. Nectar samples will be collected from bee stomachs in Eppendorf safe-lock tubes (1.5 mL).
    For **EACH SPECIES** at **EACH SITE,** the desirable minimum quantity required is **1 mL**.
15. **For each site, one sample of nectar from each species is obtained.** Any volume greater than 1 mL is also valuable, as this would allow some analytical verification. In this case, more than one Eppendorf tube can be used.
16. Remark: retain the bodies of bees in each identified jar to send to CREA (see protocol below).
17. Eppendorf safe-lock tubes will be identified with **COUNTRY CODE, CROP CODE, SITE CODE, SPECIES CODE** and **SAMPLING DATE.** Follow the labelling scheme given in WP1.1.2. (e.g. "ESP_AAP_12_B_19-04-2019"). Labels will be printed with a laser printer (to avoid smudging with humidity) and not hand written. Wrap the label around the Eppendorf.
18. Please bear in mind that the quantities of nectar are very small and that we need to prevent any nectar losses. Therefore, ensure Eppendorf tubes are **correctly closed** to avoid evaporation, and **wrap parafilm** around the Eppendorf to further seal it.
19. Nectar samples in the Eppendorf tubes should be stored in a **freezer at -20°C** as soon as possible.

20. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date, collectors and numbers of each species obtained from each hive / colony.
21. **Postage**: The samples of nectar will be posted for analyses to **ANSES**. The samples of nectar will be posted with **dry ice** with a special care to maintain the deep freeze chain (UN 1845 procedure requirements).
22. Samples will be posted with a special care to avoid crushing samples (rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements for dry ice shipments by air http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1.
23. Packages should be sent at the beginning of the week (before Wednesday) to avoid delay and blockage during the weekend.
24. For simplicity and to reduce shipment costs, each partner is recommended to organise the shipment when **ALL** of their nectar samples are collected.

Pesticide residue analyses of *Apis* and *Bombus* specimens:

1. Retain the bees used for nectar extraction for pesticide residue analyses (WP1.4.4).
2. The jars containing the bodies of specimens should be stored in a **freezer at -20°C** as soon as possible after nectar extraction on the field. The bees will be killed by being placed in the freezer overnight.
3. The specimens will be pooled to give **one sample** of *Apis* and **one sample** of *Bombus* per site.
   The bees should be transferred into **POLYETHYLENE BAGS.**
4. Maintain the cold chain until the samples arrive at the storage place. Avoid crushing samples.
5. Identify the bags with labels indicating **COUNTRY CODE, CROP CODE, SITE CODE, SPECIES, ANALYSIS CODE (NTOX)** and **SAMPLING DATE** as described in the site labelling scheme (WP 1.1.2) (e.g. "ESP_AAP_12_B_NTOX_19-04-2019").

   Look at the protocol for bee collection (WP 1.4.4) for further instructions.

6. Samples will be stored in a freezer at -20°C until posted to **CREA**.

Collecting nectar from *Apis*:

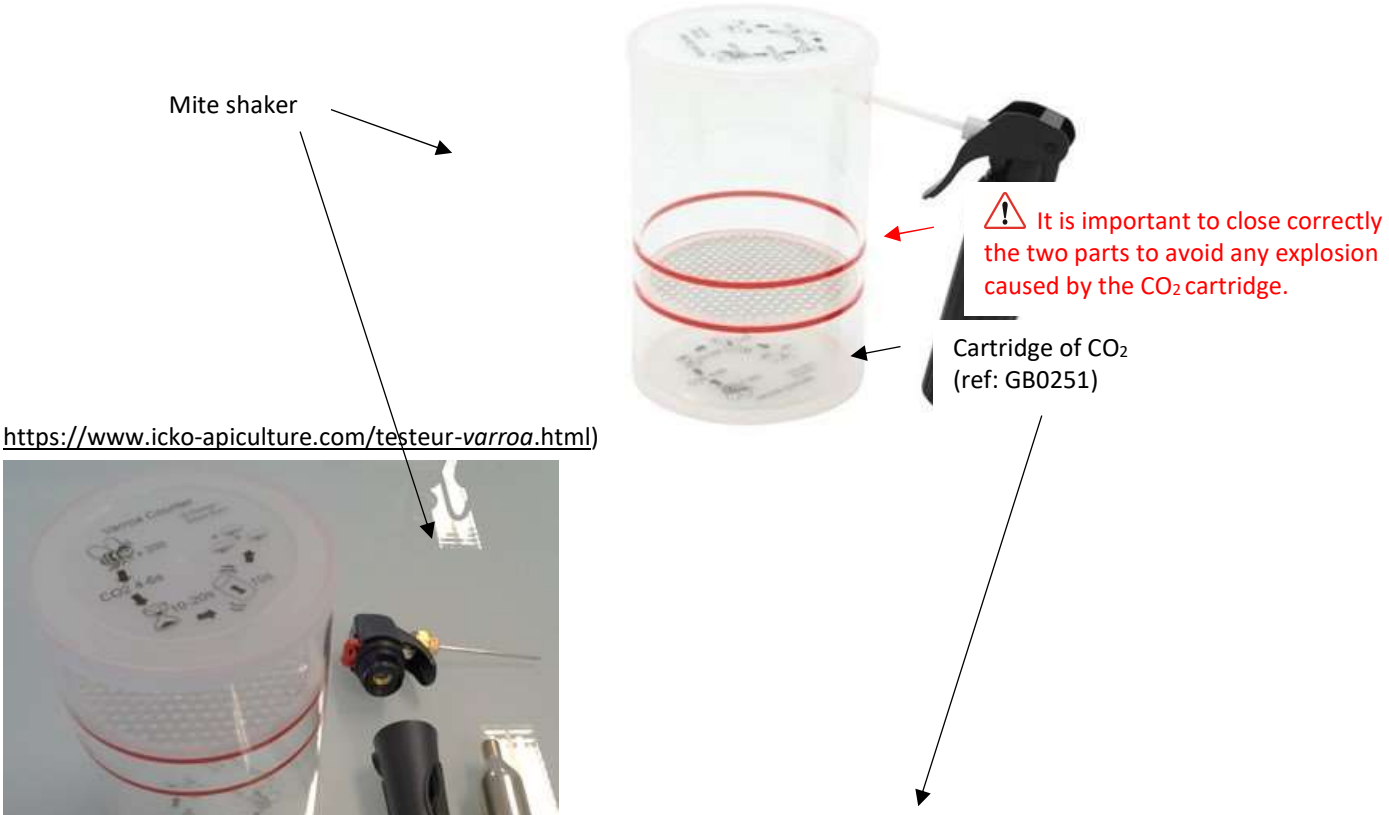
1. For ***Apis mellifera***, we require the nectar of a total **of at least 60 adult individuals** (possibly more) collected at each site: ideally this would be **20 individuals from each hive**.
2. The adult insects should be **foraging workers** returning to the hives.
3. The entrances to the hives **should be closed** so returning worker bees accumulate on the hive entrance. When collecting the bee, make sure the specimen did not regurgitate the nectar. If the bee did regurgitate, release it and collect another one. **Proceed one hive after another following the steps below.**
4. Store the bees in JARS or a CO_2 mite shaker and put the bees to sleep with CO_2 (see Figure 1 below if small cartridges of CO_2 are used and Figure 2 if CO_2 bottle is used).
5. **Be careful to close carefully the two parts of the CO_2 kit to avoid any explosion caused by the CO_2 cartridge.**
6. Once bees asleep, nectar collection should be performed as soon as possible.
7. For collecting nectar, take the bees out of the chilled container (treat again with CO_2 if necessary) and press each bee gently on the crop (honey sack) between the thumb and the forefinger (located at the beginning of the abdomen - at the level of the two first abdominal segments) (Figure 3 below).
8. The regurgitated nectar appears on the bee mandibles. Direct the drop into the Eppendorf safe-lock tube. Collect the regurgitated nectar from the bees of the **THREE** hives in the **SAME** tube labelled following the labelling scheme (e.g. "ESP_AAP_12_A_19-04-2019").
9. Eppendorf tubes should be correctly **closed and sealed with parafilm** in order to avoid evaporation. Please bear in mind that the quantities of nectar are very small and that we need to prevent any nectar losses.
10. Store the Eppendorf tubes in a jar or a bag (see below Figure 4). The jars or bags are then maintained in a chilled container.

11. Nectar samples in the Eppendorf tubes should be stored in a **freezer at -20°C** as soon as possible.
12. The bodies of the *Apis* bees will be retained and sent to **CREA** for pesticide residue analyses (WP 1.4.4). See above information on storage and postage.

Collecting nectar from *Bombus*:

1. For ***Bombus***, **foraging workers** will be collected during the crop flowering period. The aim is to collect **10 foraging workers** returning to the colony for each of the 3 colonies. However, because activity may vary between colonies and it is time consuming to collect returning workers, it is recommended to collect all returning workers independent of home colony. Note the numbers of collected foragers returning to each colony that are collected.
2. The total of **adult individuals** collected in each site should be at least **30 bumblebee workers**.
3. At start of the collection, **close the entrances** to the colonies so that returning worker bees accumulate on the colony entrance.
4. When collecting the bee, ensure the specimen did not regurgitate the nectar. If the bee did regurgitate, retain it for the specimen collection and continue to collect another one for nectar collection. **Proceed one colony after another following the steps below.**
5. Use a jar to catch bees or a hand-held insect net to catch bees that are flying around close to the entrance.
6. Store the bees in JARS or a CO_2 mite shaker and put the bees to sleep with CO_2 (see Figure 1 below if small cartridges of CO_2 are used and Figure 2 if CO_2 bottle is used). **Be careful to close carefully the two parts of the CO_2 kit to avoid any explosion caused by the CO_2 cartridge.**
7. Once bees asleep, nectar collection should be performed as soon as possible.
8. For collecting nectar, take the bees out of the chilled container (treat again with CO_2 if necessary), press each bee gently on the honey sack, by pressing the sides of the abdomen, between the thumb and the forefinger.
9. The nectar regurgitated appears on the bee mandibles. Direct the drop into the Eppendorf tube. Collect the regurgitated nectar from the bees of the **THREE** colonies in the **SAME** tube labelled following the standard labelling scheme (e.g. “ESP_AAP_12_B_19-04-2019”).
10. Eppendorf tubes should be correctly **closed and sealed with parafilm** in order to avoid evaporation: quantities of nectar are very small and that we need to prevent any nectar losses.
11. Store the Eppendorf tubes in a jar or a bag (see below Figure 4). The jars or bags are then maintained in a chilled container.
12. Nectar samples in the Eppendorf tubes should be stored in a **freezer at -20°C** as soon as possible.
13. The bodies of the *Bombus* bees will be retained and sent to **CREA** for pesticide residue analyses (WP 1.4.4). See above information on storage and postage.

Figure 1: A CO_2 kit to temporarily anesthetize the bees (*Varroa* counter type - Ref: GB025):



https://www.icko-apiculture.com/testeur-*varroa*.html)

Figure 2: A bottle of CO_2

Figure 2: Nectar extraction from a bee in an Eppendorf safe-lock tube (nectar regurgitation)

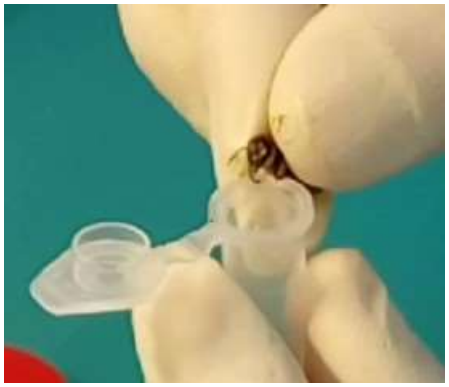


Figure 4: Storage of the Eppendorf safe-lock tubes

Summary of nectar collections for WP2

Date	Site Code	Collectors	Time	Foragers collected							
				Apis				*Bombus*			
				Hive A1	Hive A2	Hive A3	Remarks	Colony B1	Colony B2	Colony B3	Remarks
	OSR_1										
	OSR_2										
	OSR_3										
	OSR_4										
	OSR_5										
	OSR_6										
	OSR_7										
	OSR_8										
	APP_9										
	APP_10										
	APP_11										
	APP_12										
	APP_13										
	APP_14										
	APP_15										
	APP_16										

WP1.4.4 Collecting bee samples for WP2

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05.03.19

General procedure

1. The aim of this document is to describe the process for sampling adult insect pollinators for the detection of agrichemicals, metals and pathogens that form part of WP2.
2. At each site, we will collect adults of all **THREE** bee species (*Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*). In this document, hives refer to honeybees (*A. mellifera*), colonies refer to bumblebees (*B. terrestris*) and nests refer to solitary bees (*O. bicornis*).
3. At each site, the samples of adult insects will be obtained from all **THREE** hives/colonies/trap nests.
4. For ***Apis*** and ***Bombus***, the specimens used **for nectar extraction** will be sent for pesticide residue analyses at CREA (see WP1.4.3).
5. Sampling of ***Bombus*** foragers could take considerable time. The colony entrances should be closed early during the sampling visit, and bees collected as and when they arrive.
6. Numbers and timing of ***Osmia*** females returning to nests could be very sporadic. One field worker should focus on this species and collect specimens as and when they arrive.
7. Aim to collect the same number of bees from each hive / colony / nest. The differences in quantities sampled between hives/colonies/nests should be max 30%. Record the numbers collected from each hive/colony/nest in the tables below. If one hive/colony/nest does not produce the number of specimens required, increase the numbers from the remaining two in order to reach the total number requested.
8. At each site, the time points for sampling will be different for the three species due to the type of bees required per analysis and in accordance with the biology of species.
   The sampling will be performed:
   THREE TIMES for *A. mellifera*,
   THREE TIMES for *B. terrestris*
   TWICE for *O. bicornis*
9. The time points for sampling are detailed below for each species and for each analysis.
10. In the field, adult insects will be collected in polypropylene **JARS** and placed in a **CHILLED** container.
11. Be careful to identify the jars (on the cap and the container) with **COUNTRY CODE, CROP CODE, SITE CODE, SPECIES CODE, HIVE/COLONY/NEST NUMBER, ANALYSIS CODE, and DATE** as detailed in the site labelling scheme (WP1.1.2). For instance, the label "IRL_APP_13_A_PAT_15-04-2019" refers to *Apis mellifera* bees collected from site 13 of apple tree orchards in Ireland on April 15 for pathogens analysis.
12. Be careful to change gloves, collection jars, and other necessary material between bee species on the same site, and between sites, to avoid cross-contaminations.
13. Labels will be printed with a laser printer (to avoid smudging with humidity) and not hand written.
14. The samples should be stored in a freezer at -20°C as soon as possible. Please ensure to maintain the cold chain until the samples arrive at the storage place. Avoid crushing samples.
15. Fill in the **WP2 SAMPLE SUMMARY** sheets attached, with details of site, date, collectors, and numbers obtained for each species from each hive / colony / trap nest.
16. Some bees used for **haemolymph** collection (see **WP1.6.1**) will also be retained to augment these samples (especially for *Osmia* and *Bombus*). These specimens should be retained **INDIVIDUALLY** in the pre-labelled tubes supplied by the WP9 team.

Postage

1. Samples should remain in the freezer at -20°C until posted to the different WP2 partners.
2. Bees will be pooled to give **one sample** of bees for each species from each site. The bees should be transferred into **POLYETHYLENE BAGS** for storage and postage to the different laboratories.
3. Identify the bags with labels indicating **COUNTRY CODE, CROP CODE, SITE CODE, SPECIES**, **ANALYSIS CODE** and **SAMPLING DATE** as described in the **WP1.1.2 Site labelling scheme**. (e.g. "IRL_APP_13_A_PAT_15-04-2019")
   Labels should be printed with a laser printer (to avoid smudging with humidity) and not hand written (this is so the laboratories to receive samples with readable information).

4. Be aware that some labels do not fully stick after some days in the freezer. To secure the identification, there are two options:
5. Use a specific label, resistant to freezing condition: https://www.cils-international.com/en/laboratory-labels/freezer-and-cryo-labels/.
6. Use double packaging. The bag with the bees will be placed in an additional bag. The label will be placed INSIDE this **second** bag (the label should not be in contact with the bees).
7. The samples will be shipped from one institution to another with **dry ice**, taking care to maintain the deep freeze chain (UN 1845 procedure requirements). Bags will be posted to the various WP2 partners with a special care to avoid crushing samples (rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements for dry ice shipments by air http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1 .
8. Packages should be sent at the beginning of the week (before Wednesday) to avoid delay and blockage during the weekend.
9. In order to reduce shipment costs as much as possible, each partner should organise the shipment when **ALL** of their samples are collected.

COLLECTING HONEYBEES

1. For ***Apis***, workers will be collected at **three time points**:
   (1) at the installation of hives on the sites for pathogens analysis,
   (2) during the crop flowering period for metal and pesticide residue analyses,
   (3) at the end of the crop flowering period for pathogens analysis.

At the installation of hives on sites,

2. **20 internal workers** should be collected per hive (**60** honeybees per site).
3. The hives should be opened, and bees located on the outer frames should be taken.
4. Label the samples as detailed above.
5. After overnight freezing, the samples will be pooled in a single polyethylene bag per site.
6. The bags will be labelled as detailed above and posted to **ANSES Sophia Antipolis** for **pathogens analysis.**

During the crop flowering period

7. When foragers are observed on the crops it is advised to sample **30 foraging workers** per hive (**90** honeybees per site) for **metal analysis**.
8. **Close the entrances** to the hives so returning worker bees accumulate on the hive entrance. Bees can then be collected easily using a scoop, brush or cups.
9. Label the samples as detailed above.
10. After overnight freezing, the samples will be pooled and posted to the **ANSES metal lab** to the address given above.
11. On this same collection date, a further **60 foraging worker bees** per site will be collected for **nectar** sampling and then the bodies of these bees processed and posted to **CREA** for **pesticide residues analysis** (see WP1.4.3 for details)
12. **Remember to re-open the hives after sampling is completed !**

At the end of the crop flowering period

13. **20 internal workers** should be collected per hive (**60** honeybees per site).
14. The hives should be opened and bees located on border frames should be taken.
15. The samples will be pooled in a single polyethylene bag per site.
16. The bags will be labelled as detailed above and posted to **ANSES Sophia Antipolis for pathogens analysis.**
17. [**Note**: **20 internal workers** per **hive** (from the centre frames) are also required for wing asymmetry analysis as part of WP1.6.2. Field operatives may save time by obtaining these bees at the same time.]
18. [**Note**: **10 foraging workers** per **SITE** are required for gut microbiota analysis as part of WP1.6.2. Field operatives may save time by obtaining these bees during this visit]

Haemolymph samples

19. Additionally, as part of WP9, towards the end of the crop flowering period, **15 returning foraging workers** will be collected at each **SITE for haemolymph collection.** The bodies of these workers will be stored in pre-labelled tubes provided, and returned to Biopark at the address above (see **WP1.6.1** for more details).

COLLECTING BUMBLEBEES

1. For ***Bombus***, workers will be collected at **three time points**:
   (1) prior to, or during the colony being installed
   (2) during the crop flowering period for pesticide residue analyses and nectar collection,
   (3) at the end of the crop flowering period, after the colonies have been frozen.

In the lab or prior to installation of colonies on sites,

2. To pre-screen bees for pathogens, **4 *Bombus* workers** should be collected **per colony** (12 per site).
3. The doors to the colonies should be opened, and bees sampled as they emerge.

4. Pool the bees from colonies assigned to each site.
5. Label the samples as detailed in the **WP1.1.2 Site labelling scheme**: COUNTRY_CROP_SITE_B_PAT_DATE
6. Store the samples in a freezer at -20°C.
7. The bags will be posted to **ANSES Sophia Antipolis** for **pathogen analysis.**

During the crop flowering period

8. **10 returning foraging workers** should be collected per colony for **nectar collection** and pesticide residue analyses (**30** bumblebees per site).
9. **Close the entrances** to the colonies so returning forager workers accumulate on the colony entrance. Use a net, tube or jar to catch the bees at the entrance or a hand-held insect net to catch bees that are flying around close to the entrance.
10. Activity may differ between the colonies and although we should aim for an even collection between colonies, it is recommended to collect all returning foragers, regardless of their apparent home colony, until the target of 30 is reached. As this process takes time, the entrances of Bombus colonies should be closed early on during the sampling visit.
11. Label the samples as detailed above**.**
12. After nectar is collected from the specimens (see the protocol WP1.4.3), the bodies will be retained and pooled in a single polyethylene bag destined for **CREA** for **pesticide residue analyses**.
    The bag will be labelled as detailed above and posted to CREA at the given address.
13. [**Note**: **5 foraging *Bombus* workers** per **SITE** are required for gut microbiota analysis as part of WP1.6.2. Field operatives may save time by obtaining these bees during this visit]
14. **Remember to re-open the entrances of the colonies when sampling is completed!**

At the end of the crop flowering period,

15. When the *Bombus* colonies have been collected and frozen, **10 internal workers** should be collected per colony (**30** bumblebees per site).
16. Worker bees can be differentiated from queens by their smaller size and from the males by presence of pollen basket/sting sheet. Worker bees located on the brood should be taken preferentially, since bees that are lying around the sides of the colony boxes may have been dead before termination.
17. Pool the samples in a single polyethylene bag per site, and label the bag as described above.
18. These samples will be posted to **ANSES Sophia Antipolis** for **pathogens analysis**.
19. Collect 10 young-looking *Bombus* workers from each colony and place these in a plastic bag. These specimens will be used for wing asymmetry and fat body analysis and posted to **Denis Michez** (see WP1.6.2). Label accordingly (COUNTRY_CROP_SITE_B_WA_DATE) and store in freezer until posting.
20. If any *Bombus* workers remain, add these specimens to the samples for **ANSES Sophia Antipolis**. Remember to add these numbers to the data table

Haemolymph samples

20. Additionally, as part of WP9, towards the end of the crop flowering period, **5 returning foraging workers** will be collected at each **SITE for haemolymph collection.** The bodies of these workers will be stored in pre-labelled tubes provided, and returned to Biopark at the address above (see **WP1.6.1** for more details).

COLLECTING *OSMIA*

1. For *Osmia*, females will be collected at TWO **time points**:
   (1) prior to the pupae/bees being released on site
   (2) from middle to late crop flowering period

In the lab or prior to installation of nests on sites,

2. Because the *Osmia* pupae will be supplied in bulk, from the same source, to pre-screen *Osmia* for pathogens, it is only required to send **10 emerged female *Osmia*** per **COUNTRY**. Extra specimens can be sent if there are some spare.
3. These specimens can be the bees that emerge early when you are testing for emergence.

4. Label the samples as detailed in the **WP1.1.2 Site labelling scheme**: COUNTRY_CROP_SITE_O_PAT_DATE
5. Store the samples in a freezer at -20^{o}C.
6. The bags will be posted to **ANSES Sophia Antipolis** for **pathogen analysis.**

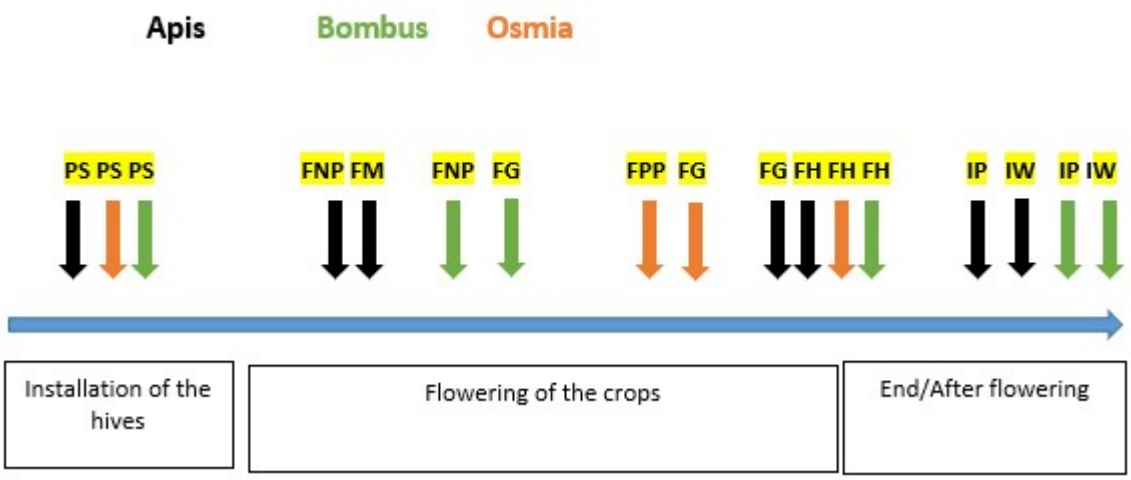
<u>During the crop flowering period</u>,

7. For ***Osmia***, foraging females will be collected during the crop flowering period for both pesticide residue analyses and for pathogens analysis.
8. Before females are collected, sufficient time is required for females to lay eggs and produce pollen stores in the trap nests. Therefore collecting should be performed from mid- to late flowering.
9. Collecting *Osmia* can be extremely time consuming, and will probably require someone to watch over the nests, collecting individuals as and when they arrive.
10. From **each site**, aim to collect **36 females** in total: **18 females** for pesticide residue analysis and **18 females** for pathogens analysis.
11. Ideally, the females would come from all three trap nests at each site. Record this information.
12. It may not be possible to collect all these specimens in one visit, so it may be necessary to collect further *Osmia* during a later visit in order to achieve the numbers of specimens required.
13. Pool the samples from each site, and label with COUNTRY_CROP_SITE_O_ TOX_DATE for the pesticide samples, and COUNTRY_CROP_SITE_O_PAT_DATE for the pathogen samples.
14. Post the specimens for pesticide residue analysis to **CREA** at the address given above**.**
15. Post the specimens for pathogens analysis to **ANSES Sophia Antipolis** at the address given above**.**
16. [If possible, once all these specimens are collected, and *Osmia* have been processed for haemolymph analysis, collect up to 5 extra *Osmia* foragers for Gut Microbiota analysis (WP1.6.2)]

<u>Haemolymph samples</u>

17. Additionally, as part of WP9, towards the end of the crop flowering period, **5 returning females** will be collected at each **SITE** for **haemolymph** collection**.** The bodies of these workers will be stored in pre-labelled tubes provided, and returned to Biopark (see **WP1.6.1** for more details).

Summary for bee collection at each site



PS: Pre-screening bees for pathogens
FNP: Forager workers for nectar collection and pesticide residue analyses
FM: Forager workers for metal analyses
FPP: Returning females for pathogens and pesticide residue analyses
FH: Foraging workers/ females used for haemolymph collection
FG: Foraging females for gut microbiota analyses
IP: Internal workers for pathogens analysis
IW: Internal workers for wing asymmetry/fat body analyses

Summary of *APIS* specimen collections for WP2

Hive installation - internal workers – pre-screening for pathogens analysis

			Hive		
Site code	**Date**	**Collector(s)**	**A1**	**A2**	**A3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Foraging workers - metal analysis

			Hive		
Site code	**Date**	**Collector(s)**	**A1**	**A2**	**A3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Foraging workers - Nectar / Chemical residue analyses

			Hive		
Site code	**Date**	**Collector(s)**	**A1**	**A2**	**A3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

End of flowering period - internal workers for pathogens analysis

			Hive		
Site code	**Date**	**Collector(s)**	**A1**	**A2**	**A3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Summary of *BOMBUS* specimen collections for WP2

Pre-screening for pathogens analysis

			Colony		
Site code	**Date**	**Collector(s)**	**B1**	**B2**	**B3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Foraging workers - nectar / pesticide residue analyses

			Colony		
Site code	**Date**	**Collector(s)**	**B1**	**B2**	**B3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

End of flowering period - internal workers after box is frozen for pathogens analysis

			Colony		
Site code	**Date**	**Collector(s)**	**B1**	**B2**	**B3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Summary of *OSMIA* specimen collections for WP2

Returning females for pesticide residue and pathogens analyses

			Colony		
Site code	**Date**	**Collector(s)**	**O1**	**O2**	**O3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

(note extra copies of this table may be needed if specimens are obtained on different dates from the same site)

WP1.4.5 Collecting beebread / stored pollen samples for WP2

T Kiljanek, M Laurent, M Albrecht, M Rundlöf, S Hodge

06.03.2019

General

1. The aim of this process is to collect beebread/pollen stores samples that will be used in the analysis of agrichemicals, nutrition quality and palynology that form part of WP2.
2. At each site, beebread/pollen stores of all **THREE** bee species (*Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*) will be collected. Details for collecting method for each species are given below.
3. Hives refer to honeybees (*A. mellifera*), colonies refer to bumblebees (*B. terrestris*) and nests refer to *Osmia.*
4. Beebread/pollen stores should be obtained from all **THREE** hives/colonies/nests at each site in equal proportions to standardize the material tested by all laboratories.
5. The sampling should be performed only **ONCE** for each species at each site.
   For *Apis* and *Bombus* the sampling should be performed towards the end of the flowering period.
   For *Osmia*, the sampling should be performed at the peak crop bloom activity.
6. Extracted beebread/ pollen stores samples will be **POOLED** per site.
7. Store the samples in polyethylene bags or polypropylene jars resistant to freezing.
8. Samples should be identified with a **pre-printed label**, resistant to freezing, with **COUNTRY CODE, CROP CODE, SITE CODE, SUBSTANCE CODE** and **SAMPLING DATE** (see **WP1.1.2 Site labelling scheme).**
   Use **APS** for *Apis* Pollen Stores; **BPS** for *Bombus* Pollen Stores; **OPS** for *Osmia* Pollen Stores
   (e.g. "IRL_APP_13_OPS_15-04-2019")
9. Be aware that some labels do not fully stick after some days in the freezer. To secure the identification, there are two options:

   Use a specific label, resistant to freezing condition: https://www.cils-international.com/en/laboratory-labels/freezer-and-cryo-labels/.

   Use double packaging. The bag with the bees will be placed in an additional bag. The label will be placed INSIDE this **second** bag (the label should not be in contact with the bees).
10. Be careful to change gloves, collection jars, and other necessary materials between bee species on the same site, and between sites to avoid cross-contaminations.
11. Samples should be stored in a **freezer** below **-20ºC** until posted to PIWET.
12. **Post the whole sample to PIWET** with **dry ice,** taking care to maintain the deep freeze chain.
13. PIWET will homogenize received samples.
14. PIWET will prepare sub-samples and post them to UMONS and CREA.
15. The following **MINIMUM QUANTITIES** of **extracted beebread/pollen stores** from each site are required for analysis of:

Pesticides, metals, glyphosate (PIWet)	12.0 g
AA &sterols (UMONS)	0.1 g
Palynology (CREA)	3.0 g

16. These quantities should be considered as the **minimum** requirement, and the aim should be to prepare sub-samples with more weight where possible.

Collecting beebread of *Apis*

In the field:

1. Pieces of comb should be cut out from all **three** hives at each site.
   Aim to collect a minimum quantity of 5 g per hive.
   Collect several pieces of comb from different frames in order to obtain a representative sample.
   This process could take 30 mins per site.
2. The pieces of comb should contain cells filled out with beebread.
3. The honeycomb will be stored in **PLASTIC (polyethylene) BAGS** clearly labelled as described in the WP1.1.2 Site labelling scheme.
4. Place the bags in a **CHILLED** container. Please avoid crushing samples. The samples should be placed in a **FREEZER** as soon as possible (* notice that polyethylene is resistant to freezing).
5. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date and collectors.

At the home institute:

6. Beebread should be **EXTRACTED** from cells, separately from each hive. It takes one minute to extract 1 g of beebread from cells.
7. Beebread should be extracted from cells using a **STAINLESS STEEL** spatula (e.g. ROTH art no. AT18.1)

8. Extracted beebread should be **POOLED** from all three hives in equal proportions.
9. The weight of each pooled sample should be recorded in the comments section of the sheet attached.
10. A **MINIMUM** required quantity of pooled sample to conduct all analyses is **15.1 g** from each site.
    The aim should be to prepare samples with more weight where possible.
    If, however, the sample has a lower weight, it should also be sent to the laboratory to perform at least some part of analyses.
11. Store the extracted beebread in a polypropylene jar identified with a **pre-printed label** (**WP1.1.2 Site labelling scheme)**.
12. Samples should be stored in a **freezer** below **-20°C** until posted to PIWET.

Remark: change the materials (gloves, stainless steel spatula) between each site.

Link to video showing beebread extraction from comb

https://drive.google.com/file/d/1L0Z8jp72ibwFIgnu1q4SOVyL9s7Fz0Kg/view

Collecting pollen stores of *Bombus*

In the field:

1. Pollen from *Bombus* foragers that are sampled for nectar and pesticide residue analyses will be sampled during the flowering season and stored (see 1.4.2).
2. At the end of the flowering season the *Bombus* colonies will be terminated by freezing (see 1.5.9).
   Pollen stores will be sampled from these frozen colonies.
   The pollen is stored in the colony in open isolated cells (see picture).
3. The *Bombus* colonies will be stored in **PLASTIC (polyethylene) BAGS** clearly labelled as described in the WP1.1.2 Site labelling scheme.
4. Colonies should be placed in a **FREEZER** as soon as possible.

At the home institute:

5. All stored pollen should be extracted from cells and **POOLED** from all three colonies into a pre-weighed plastic container. Pollen stores can be extracted from storage cells using a **STAINLESS-STEEL** spatula.

6. The weight of pollen from each colony can be recorded in the comments section of the sheet attached.

7. A **MINIMUM** required quantity of pooled sample to conduct all analyses is **15.1 g** from each site.

8. The aim should be to prepare samples with more weight where possible.
If, however, the sample has a lower weight, it should also be sent to the laboratory to perform at least some part of analyses.

9. Store the extracted pollen stores in a polypropylene jar identified with a **pre-printed label** (see point 8 in General).

10. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date and collectors, weight of the empty plastic container and weight of the pollen (weight of the container with pollen subtracting the weight of the empty container).

11. Samples should be stored in a **freezer** below **-20°C** until posted to PIWET.

Collecting pollen stores of *Osmia*

1. Around the peak of the flowering season of the focal crop, approximately **10 *Osmia* nest tubes** (single cardboard tubes) will be collected from **each site.** Try to use roughly equal numbers of tubes from each nest.

2. The nest tubes must be collected **before** the larvae eat all the stored pollen.
Thus, it is better to collect those cardboard tubes that have just recently been closed with mud at the entrance at the front of the tube.

3. Pull the cardboard tube carefully out from the trap nest: using forceps is recommended.
Replace with a new, empty cardboard tube (these will be included in your *Osmia* kits).

4. Write on the tube, the CROP CODE, the SITE CODE, the nest number and the SAMPLING DATE
Store in a **PLASTIC** (**polyethylene) BAG** with other tubes only from that site.
Place the plastic bag in a **CHILLED** container until processing.
The tubes should be placed in a **FREEZER** as soon as possible.

5. Fill in the **WP2 SAMPLE SUMMARY** sheet attached, with details of site, date and collectors.

At the home institute:

6. Split the nest tube can in half using a sharp knife or scalpel.
   Count the number of **cells**, **eggs** and **larvae** in each tube and record this in the **WP2 SAMPLE SUMMARY** sheet attached. Remove eggs and larvae.
7. Collect the pollen stores from each tube using a clean stainless-steel spatula.
8. Extracted pollen stores should be **POOLED** from all nest tubes in equal proportions.
9. The weight of the pollen stores collected in each tube **AND** the weight of each pooled sample should be recorded in the table provided.
10. If possible, try and ensure that only nests actually constructed by *Osmia bicornis* rather than any other *Osmia* or trap-nesting bee species are sampled for pollen stores.
11. A **MINIMUM** required quantity of pooled sample is required to conduct all analyses is **15.1 g** from each site.
12. The aim should be to prepare samples with more weight where possible.
    If, however, the sample has a lower weight, it should also be sent to the laboratory to perform at least some part of analyses.
13. Store the extracted pollen stores in a polypropylene jar identified with a **pre-printed label** (**WP1.1.2 Site labelling scheme)**.
14. Pollen stores should be stored in a **freezer** below **-20°C** until posted to PIWET.

Postage

1. The samples will be shipped from one institution to another with **dry ice**, taking care to maintain the deep freeze chain (UN 1845 procedure requirements). Samples will be posted to PIWET with special care to avoid crushing (use rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements for dry ice shipments by air
   http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1
2. Packages should be sent at the beginning of the week (before Wednesday) to avoid delay and blockage during the weekend.
3. To reduce shipment costs as much as possible, each partner is recommended to organise the shipment when **ALL** of the samples are collected.

Summary of beebread/pollen stores collections for WP2

Country code:

	Site code	Date collected	Weight empty container (g)	Pollen weight (g)	Collector(s)	Comments
Apis	OSR_1					
	OSR_2					
	OSR_3					
	OSR_4					
	OSR_5					
	OSR_6					
	OSR_7					
	OSR_8					
	APP_9					
	APP_10					
	APP_11					
	APP_12					
	APP_13					
	APP_14					
	APP_15					
	APP_16					
Bombus	OSR_1					
	OSR_2					
	OSR_3					
	OSR_4					
	OSR_5					
	OSR_6					
	OSR_7					
	OSR_8					
	APP_9					
	APP_10					
	APP_11					
	APP_12					
	APP_13					
	APP_14					
	APP_15					
	APP_16					

Osmia stored pollen collection table for each tube

Date	**Site**	**Nest code**	**Cell count**	**Larvae/ egg count**	**Pollen weight (g)**	**Total pollen weight this site**

WP1.4.6 Collecting bee wax (*Apis* & *Bombus*)

Gennaro Di Prisco, Joachim de Miranda, Maj Rundlöf, Cecilia Costa, Piotr Medrzycki

05.03.2019

General procedure

1. The aim of this process is to collect samples of bee wax that will be used in the analysis of agrochemicals.

2. Bee wax will **only** be collected in **Sweden**, **Italy** and **Germany**. The other WP1 partners can ignore this protocol.

3. Samples of bee wax will be collected only **once**, from **4 OSR** and **4 APP** sites, preferably the same sites used for Royal Jelly collection (see protocol WP1.4.7).

4. The wax samples should be produced **during** the flowering period.

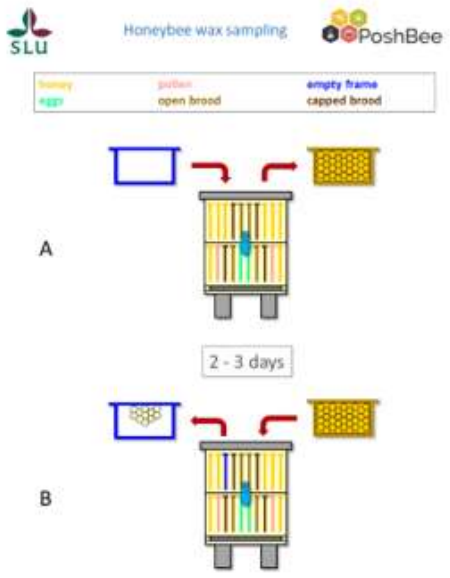
5. At each site, bee wax will be collected from **all three** *Apis mellifera* beehives and from **all three** *Bombus terrestris* colonies.

6. A minimum of **2 g CLEAN** wax should be collected **per hive/colony**.
The samples will be pooled per site and per bee species, so each site will deliver at least **6 g** honeybee wax and **6 g** bumblebee wax.
The exact weight is not important, other than that similar amounts are collected from each colony.

7. For honeybee colonies it is easy to produce clean wax (see below), but for bumblebee colonies it may be more difficult to guarantee that the wax is free of brood, nectar or pollen. In this case, more wax needs to be collected to compensate.

A. Honeybee wax production

1. During the second week of bloom, replace a honey frame next to the brood cluster with an **empty** frame (*i.e.* no comb or foundation). This is where normally drone brood is produced in the nest and a normal-size colony should draw a lot of drone-wax here very quickly, especially during swarming season with a strong nectar flow.
2. Return after 2-3 days to the check wax production. If enough wax has been produced (2 g is about 10 cm x 10 cm new comb), gently **break off** the wax cake with gloved hands, or **cut off** using a **clean** knife (i.e. **NOT** a hive tool) and return the original honey frame to its position in the hive. If possible, choose the portion of wax cake which does not contain brood, honey or pollen.
3. If not enough wax has been produced, leave the frame for another few days and harvest then. See figure 1.



B. <u>Bumblebee wax production</u>

1. To ensure collection of clean wax, avoid placing cotton wool in the colonies in the 4 sites chosen for wax collection, or remove any cotton wool inserted by the bumble bee producer.
2. The bumblebee wax produced **<u>DURING</u>** the flowering period will be collected **<u>AFTER</u>** the flowering has finished and the bumblebee colonies have been frozen for dissection and analysis. Coordinate dissection procedure with **WP1.4.5 Collecting bee bread/stored pollen** and **WP1.5.9 Assessment of *Bombus terrestris* colony performance & natural enemies.** These protocols also have images and videos of how to dissect a bumblebee colony.
3. If the colony produced a wax cover (Fig. 2), use this as the wax sample. Otherwise, collect the newest wax, which is the wax covering the egg clusters and young larvae. These cells will furthermore be uppermost in the colony and the first ones to be dissected for analysis. Avoid collecting wax from older larvae as this is not separable from the cocoons and difficult to analyse. See Figure 3.
4. Remove the wax from the uppermost cups/cocoons. If the content is eggs or young larvae, save the wax. Repeat dissecting young cells and registering the content, and keep collecting this wax until 2 g has been collected.
5. Continue with the dissection of the bumblebee colony for other samples/protocols.

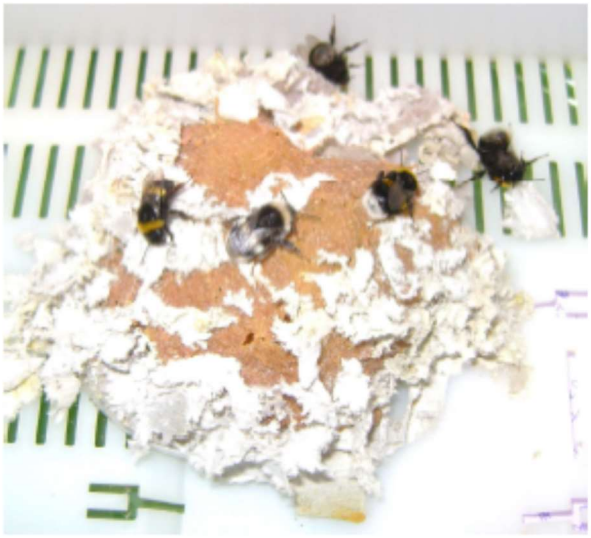


Figure 2. Example of a wax cover in a small bumble bee colony. In this case it was mixed with paper, but a certain amount of wax is clean. Photo by Laura Bortolotti and Gherardo Bogo.

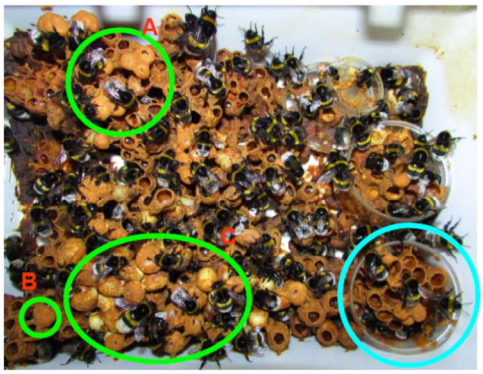


Figure 3. *Bombus terrestris* nest with indication of cells suitable for wax collection.

A: larvae young enough to collect the wax that makes up their cells for analysis.

B and blue circle: cells with eggs or very young larvae – wax can be kept for analysis.

C: older spinning larvae, the amount of wax is not sufficient and difficult to separate from their silk.

The blue circle shows newly built cells, in containers where pollen was provided (lab conditions).

Photo by Laura Bortolotti and Gherardo Bogo.

C. Sample collection & management

1. For each site, pool the three honeybee wax samples in a single **polyethylene** bag.
2. Similarly, when dissecting the bumblebee colonies (after the bloom has finished and the colonies have been collected and frozen) pool the three bumblebee wax samples from the site in a **single** polyethylene bag.
3. Identify the bags with **DATE** and **SITE CODE** and **SUBSTANCE**.
   Place in a **chilled** container until the samples arrive at the storage facility. Follow the agreed printing standards and labelling schemes for labelling the samples.
4. Bring the samples to a cold storage facility within the day of collection and transfer them to -20°C until sending them to CREA and other WP2 partners for analysis.
5. The labels should be printed with a laser printer (to avoid smudging with humidity) and not hand written (this is so the laboratories to receive samples with readable information).
6. **Postage.** The samples will be shipped between institutions on **dry ice**, taking care to maintain the deep freeze chain, using the fastest service possible (3 days maximum). Bags will be posted to the various WP2 partners with a special care to avoid crushing samples (rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements (UN 1845)
   http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1 .
7. In order to facilitate the logistics and reduce shipment costs, each partner should organise their shipment when **ALL** of their samples have been collected, *i.e.* wax, honey, pollen, beebread, royal jelly, bees *etc.*.

Summary of beeswax collection for WP2
Country code:

Sample-ID	**Species**	**Site code**	**Date(s)**	**Collector(s)**	**Total fresh weight (g)**
	Apis				
	Bombus				

WP1.4.7 Collecting Royal Jelly for WP2 (*Apis*)

J de Miranda, G di Prisco

05.03.2019

General procedure

1. The aim of this protocol is to describe the process for sampling royal jelly from honeybee hives for the analysis of specimens for agrochemicals that form part of WP2.

2. Royal Jelly will **only** be sampled in **Sweden**, **Italy** and **Germany**.
The other WP1 partners can ignore this protocol.

3. Royal Jelly will only be collected **once**, from **4 OSR** and **4 APP** sites; preferably the same sites used for wax collection (see protocol WP1.4.6).

4. At each field-site, at least 5 queen cells (10-15 will be the best) will be collected. The queen-cells should ideally be **recently** closed, to maximise the amount of Royal Jelly collected.

5. The queen-cells can be collected from **any and all** of the hives at a site: they don't have to be collected all from the same colony.

6. The queen-cells will be sampled during the routine swarm checks across the OSR and apple blooming periods, which generally coincide with the swarming season (**A**).
Additionally, a method is given to set up **one** 'Demaree' colony per site to produce many queen-cells without splitting the colony or losing productivity (**B**).

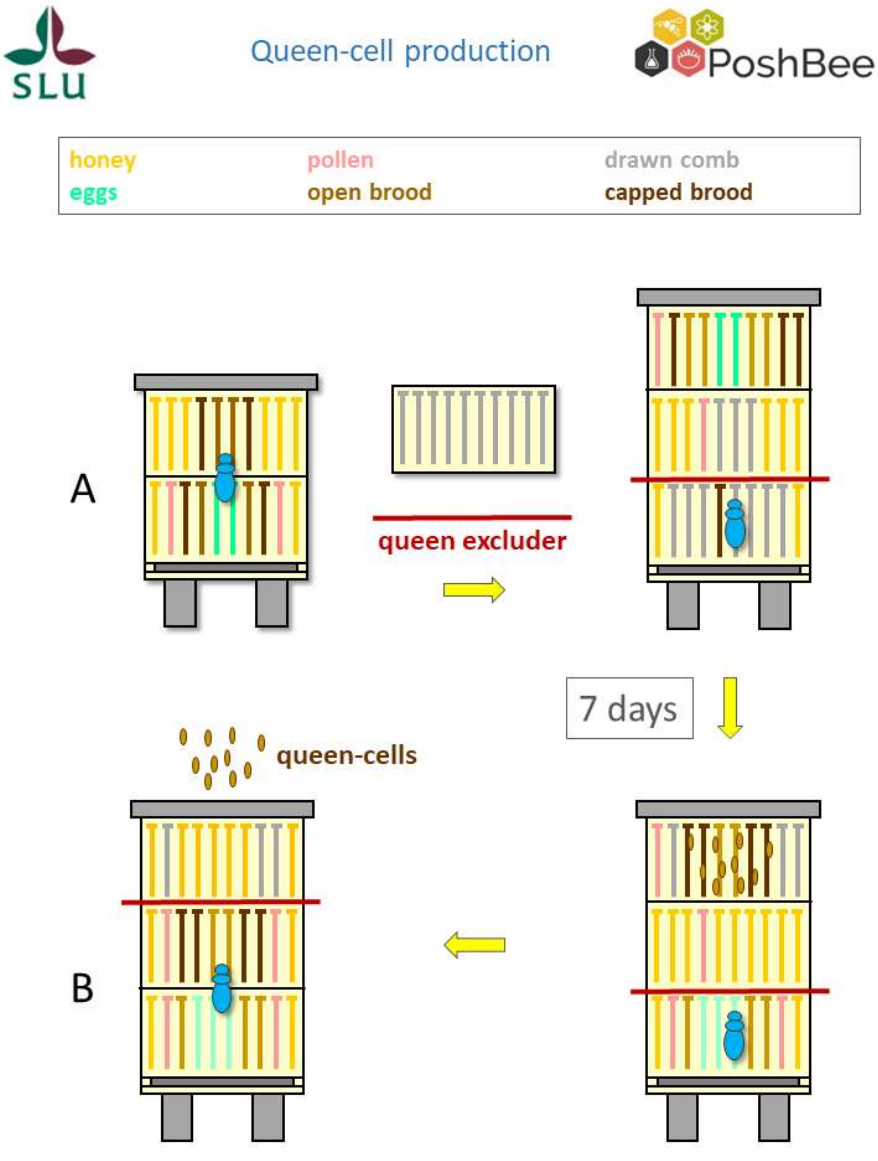
Natural queen-cells

7. During regular colony inspections, take note of the presence, location and developmental stage of newly started queen-cells.
8. From the developmental stage, estimate when the queen-cell should be nearly closed. This will require a little prognostic calculating from your cooperating beekeeper.
9. Return on the calculated day to collect the queen cell.
10. Implement swarm management on the colonies producing swarm-cells, through regular subsequent inspections and removal of queen-cells.

Demaree swarm control & queen-cell production

11. If at a particular field-site, no swarm cells are produced naturally during the first **two weeks** of the bloom period, we can stimulate swarm cell production during the **last** week of the bloom. This method also serves as a means of swarm control, without losing queen productivity (e.g. without influencing the natural colony growth dynamics and parameters) and would therefore be useful to apply **both** in case of queen-cells (to prevent the colony from swarming) **and** in the case of no queen-cells (to produce queen-cells, for collecting). Start this method immediately **after** the wax collection has finished (in any case, not before wax collecting has finished) (WP1.4.6).
12. The principle of the method is to separate the queen from the brood, using boxes of honey/frames, so that the bees with the brood think they are queen-less, while the queen has a lot of fresh comb to lay in and a smaller 'colony' of bees. The method is outlined in Figure 1 below.
13. Equipment needed:
14. A colony on two boxes, preferably one with no more than 10 frames of brood (capped brood/open brood/eggs).
15. 1 queen-excluder
16. 1 box with dry drawn comb frames and/or foundation frames
17. Locate the queen, and store her temporarily in a queen-cage.
18. Re-arrange the frames so that as much of the brood goes into a single box, and **definitely** all open brood. If there are more than 10 brood frames, keep the fully capped brood separate.
19. Fill the bottom box with drawn comb, any left-over capped brood, and a few honey/pollen frames.
20. Release queen in the bottom box, add a queen-excluder on top of the bottom box.
21. Place on top of the queen-excluder a full box with honey frames or empty drawn comb.

22. Place the box with all the brood on top of the second box. The distance between the brood and the queen means the bees here think they are queen-less, and will prepare queen cells.
23. After **7 days** (no more, no less), collect **ALL** the queen-cells in the top box. There should not be any queen-cells in the bottom box, where the queen is, or the middle box (honey and drawn comb).
24. **!!! DO NOT LEAVE ANY QUEEN-CELLS BEHIND !!!** Else you will have a re-queening event, and this would be disastrous for the experiment. Make sure **ALL** queen-cells are removed. Return within 1 week to check for any left-over cells that might have been missed.
25. Insert the top box in between the bottom box and the queen-excluder, so as to re-unite the queen with her colony and brood. Meanwhile the colony will think it has gone through a successful swarming cycle, and be less inclined to swarm again.
26. Since these manipulations are controlled by the beekeeper, the production, maturation and harvesting of the queen-cells will be much easier to organize than natural queen-cells.
27.



D. Queen-cell transport, Royal Jelly collecting, transport, storage and labelling

1. Collect the queen-cells (natural or Demaree) in labelled polyethylene bags and transport to the field laboratory on ice.
2. At the laboratory, remove with a spatula the Royal Jelly from all the queen-cells collected at a particular site, and transfer this to a polypropylene tube, pooling the Royal Jelly per site.
3. Fill in the Royal Jelly collection summary table below.
4. Identify the tube with the **DATE, SITE CODE** and **RJ**, using the agreed label printing methods and labelling scheme (**WP1.1.2 Site labelling scheme**). Labels should ideally be printed with a laser printer (to avoid smudging with humidity) and not hand written (so that the laboratories receiving the samples can read the information).
5. Samples will be stored in a freezer at - 20°C until posted to the different WP2 partners (CREA) for analysis.
6. **Postage.** The samples will be shipped from one institution to another with **dry ice**, taking care to maintain the deep freeze chain. Bags will be posted to the various WP2 partners with a special care to avoid crushing samples (rigid packaging). In case packages are posted using airplane, please respect IATA procedure requirements (UN 1845) http://www.who.int/ihr/biosafety/module_vi_shipping_dry_ice.pdf?ua=1 .
7. In order to reduce shipment costs as much as possible, each partner should organise the shipment when **ALL** of their samples are collected.

Summary of *Apis* Royal Jelly collection for WP2

Country code:

				Demaree	
Sample code	**Site code**	**Date(s)**	**Collector(s)**	**colony ID**	**Total Fresh Weight (g)**

WP1.5.0 Overview: *Apis* pests and pathogens

S Hodge, I Bottero

06/03/2018

1. We will perform multiple assessments of hive 'strength', disease symptoms and the presence of invertebrate pests in the field.
2. We suggest the presence of the beekeepers would be of great assistance when performing these assessments.
3. We will assess:
   a) Hive 'strength' (WP1.5.1)
   b) *Varroa* mite infestation (WP1.5.2)
   c) Small Hive Beetle (SHB) (WP1.5.3)
   d) *Vespa velutina* (Asian hornet) (WP1.5.4)
   e) Chalkbrood (WP1.5.5)
   f) American Foulbrood (AFB) (WP1.5.6)
   g) European Foulbrood (EFB) (WP1.5.7)
   h) Dwarf Wing Virus (DWV) (WP1.5.8)
4. Methods for each of the above hive assessments have been provided as separate documents, outlined above.
5. The hive strength based on Col Eval could take considerable time at each site.
6. However, many of these assessments can/ should be performed at the same time.
7. For example, we would advise performing assessments of *Varroa* and SHB at the same time, as these assessments will take more than visit to the same site.
8. Similarly, chalkbrood, AFB, EFB and DWV assessments should be performed concurrently, and could be during the final hive strength evaluation.
9. The presence of *V. velutina* is rapid (< 5 min) and could be scored on multiple visits.
10. Tables have been provided with each protocol for scoring the associated data.
11. SHB and AFB are notifiable pests under EU regulations.
    Your national regulations may also require you to report the occurrence of other pests.
    eg in UK all brood diseases should be reported to your local beekeeping inspector, and *Vespa velutina* should be reported to the Non-native Species Secretariat.
    Please examine your national rules governing the pests and pathogens we will record for Poshbee.
    If in doubt, inform your local beekeeping organisation and seek their advice.

WP1.5.1 Colony strength evaluation

A Knauer, S Hodge

06/03/19

- Colony strength will be assessed subjectively following Delaplane et al. (2013) and Sandrock et al. (2014). The method is based on a human observer, who visually estimates the surface area of each comb covered with bees, worker brood and pollen (as an indication of food availability).
- The assessment should be performed **TWICE**: once before and once after the flowering period.
- Time will be saved if the colony strength evaluation is performed simultaneously with other work requiring the opening of the hive.
- The assessment should be performed by, or in collaboration with, an experienced beekeeper.
- Record the data for each hive in the table provided.
- A related Excel sheet will also be provided if researchers want to directly enter data into a digital format.

Estimation of number of bees, brood area and pollen amount

1. Visual estimates of bees on combs will vary according to time of day and bee foraging activity. For this reason it is important to control for this effect by limiting observations to time windows with similar foraging activity (similar weather and day periods).
2. Also, estimates should be carried out by the same observer per crop.
3. To record data, it is best to carry out the estimates together with a partner that notes the estimated numbers.
4. To measure colony strength, colonies are opened and each comb is removed sequentially. If supers are present, these are estimated first. If there are two brood chambers, the upper one is estimated before the lower one.
5. All estimates are made to the nearest 5%. The total area always refers to the inner border of the wooden frame. Both sides per comb are estimated separately.
6. The observer looks at one side of a comb and visually estimates the percentage of the comb surface covered by bees, then turns the comb and estimates the percentage of bees on the second side. These estimates should be done quickly as bees tend to move a lot and walk from one side of the frame to the other.
7. Afterwards the observer estimates the percentage of surface covered by:
   i) sealed **worker** brood (**drone brood is not estimated**)
   ii) pollen storage
8. If it is not possible to see the brood/pollen because the whole comb is covered with bees, these can be brushed away **to the frames that have already been estimated**.
9. Combs are labelled with continuous numbers; comb sides are referred to as A and B. So, 2A refers to the first side of the second comb.
10. **OPTIONAL:** To facilitate estimation “assistant-frames” can be used that are divided into 10 equal pieces by wire (Figure 2). These “assistant-frames” can be hold above the comb of interest and allow the observer visualizing the area that represents 10% of the total comb area. These “assistant-frame” need to be prepared by each group itself as comb formats differ between countries. If in your hive system the format of the supers differs from the brood chamber, you will need to prepare two different “assistant-frames”.
11. Estimation of the percentage of surface covered by bees, works best by mentally pushing bees together into a continuous mass. It is important to visually sort the bees into a contiguous mass that approximates their density if the frame were fully covered (Fig 3) as the number of bees in the hive will later be calculated based on such a density.
12. To be able to calculate the total number of bees and brood cells per comb later, the length and width of the frames in use need to be noted.
13. **TRAINING:** To ensure precise measurements of colony strength, each observer needs to practice estimating percentage of surface covered by different compartments. The training is done on the webpage http://w3.avignon.inra.fr/lavandes/biosp/coleval.html. There are different training modules available; in each of them estimates of different compartments need to be done. At the end of each module the estimation precision of the observer is evaluated. Each observer needs to go **through all the modules until the estimation error is below 10% in each module.**

Important: By end of February 2019 two new modules will be uploaded which are based on pictures of combs. These need to be practiced as well**.** Unfortunately, the homepage is available in French only. However, as it is based on visual training, very limited vocabulary is needed. The relevant words are:

Bleu foncé = dark blue

Bleu ciel = light blue

Jaune = yellow

L'abeilles = bees

Couvain = brood

Presence of queen

Record whether the queen is present. If you cannot find the queen, note if eggs are present (as larvae hatch 3 days after eggs have been laid, eggs indicate the queen's presence during the last 3 days).

Also record the age of the queen (your beekeeper can help with this)

Indication for swarming

During the swarming process of honey bee colonies about 50% of the bees are leaving the hive. Swarming can therefore strongly affect measures of colony development. Therefore, record the presence of queen cells during colony assessment.

Pests and pathogens

To save time, it may be possible to perform pest and pathogen screening as outlined in WP1.5.2-1.5.8 for chalkbrood, EFB, AFB, DWV during the **SECOND** colony strength evaluation.
Record these results in the separate tables provided for each protocol.

References

Delaplane KS et al (2013) Standard methods for estimating strength parameters of *Apis mellifera* colonies, Journal of Apicultural Research, 52:1, 1

Sandrock C et al (2014) Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure, PLOS ONE, 9:8, e103592

Date_____ **Start Time**_____ **Site**_____ **Hive**_____

Observers_____ **Weather**_____

Queen present_____ **Queen age**_____ **Eggs present**_____ **Queen cells present**_____

Brood chamber length/width_____ **Super frame length/ width**_____

Super	Upper brood chamber		Lower brood chamber	
	Comb		Comb	
% area bees		% area bees		% area bees
% area sealed worker brood	1A	% area sealed worker brood	1A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	1B	% area sealed worker brood	1B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	2A	% area sealed worker brood	2A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	2B	% area sealed worker brood	2B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	3A	% area sealed worker brood	3A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	3B	% area sealed worker brood	3B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	4A	% area sealed worker brood	4A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	4B	% area sealed worker brood	4B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	5A	% area sealed worker brood	5A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	5B	% area sealed worker brood	5B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	6A	% area sealed worker brood	6A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	6B	% area sealed worker brood	6B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	7A	% area sealed worker brood	7A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	7B	% area sealed worker brood	7B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	8A	% area sealed worker brood	8A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	8B	% area sealed worker brood	8B	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	9A	% area sealed worker brood	9A	% area sealed worker brood
% area pollen		% area pollen		% area pollen
% area bees		% area bees		% area bees
% area sealed worker brood	9B	% area sealed worker brood	9B	% area sealed worker brood
% area pollen		% area pollen		% area pollen

WP1.5.2 *Varroa* mite infestation

S Hodge, I Bottero, E Attridge, A Knauer

25/01/2019

1. To **quantify** levels of *Varroa* infestation, we will use **examination of debris** in **each hive** based on a method commonly used by beekeepers (see also Dietemann et al. 2013).
2. Adult female *Varroa* are oval, flat, and red-brown in colour (1-1.6 mm long).
   Males are lighter and smaller, as are juvenile mites (beeaware.org.au/archive-pest/*varroa*-mites/).
   In this assessment we will **ONLY** count **ADULT FEMALES**
3. Familiarize yourself with the appearance of *Varroa* before carrying out assessments;
   Do not confuse *Varroa* with other mites that may be present.
4. Hives must be equipped with a **bottom board**.
5. The board must be protected by a **mesh** which allows the mites to fall through but prevents bees access, otherwise bees will discard the dead mites from the hive.
6. *Varroa* assessment can take place from the **middle** of flowering; if field workers are running short of time, assessment could occur at the **end** or **after** flowering of the target crop, as time allows.
7. Researchers may benefit from the assistance of the beekeeper in performing *Varroa* assessment (as with other hive disease / pest diagnostics).
8. To save time, the *Varroa* assessment should take place at the same time as the **SHB** assessment using 'beetle blasters' (WP1.5.3)
9. Initially, the hive bottom board should be **cleaned** of all debris.
10. A **yellow sticky trap** (cut to cover **the whole** bottom board) should be inserted onto the bottom board.
    Record the length (L) and breadth (B) of the sticky trap in the table below in cm.
11. After several days have elapsed, the sticky traps should be removed.
    A **minimum of 3 days** should have elapsed between set up and removal of traps.
12. **Adult female** *Varroa* mites can be counted directly on the sticky traps in the field.
13. There will be lots of other debris on the sticky trap: pollen, propolis, bits of insects, other pests etc.
    In case a large quantity of debris prevents easy detection of mites, the boards can be covered in plastic ("cling") film and later examined in the **laboratory**.
14. Data for **each hive** should be recorded in the table below.
    (In the final data set infestation rates will be expressed as '**mites per cm^2 per day'.**
15. Remember to change gloves after handling sticky traps, and be careful to avoid spreading mites when transporting used traps between sites.
16. Beekeepers may be able to detect the presence of *Varroa* mites on brood or on adult bees.
    This should be noted in the table below.
17. Also, as mite counts will be strongly influenced by previous *Varroa* treatments, this information should be retrieved from the beekeeper and added to the table below.
18. There are many videos on you tube of beekeepers using a base board for *Varroa* assessment:
    https://www.youtube.com/watch?v=q5EkmzOVUlY
    https://www.youtube.com/watch?v=YxK_lKtRuBE
    https://www.youtube.com/watch?v=pYYTdgmLJ5I

Varroa on *A. mellifera* larvae (photo by E. Attridge)

References

Dietemann et al. (2013) Standard methods for *varroa* research. J Apic Res 52:1, 1-54, DOI: 10.3896/IBRA.1.52.1.09

***Varroa* mite results table**

				Hive A1			Hive A2			Hive A3		
				L x B (cm)			L x B (cm)			L x B (cm)		
Site	Beekeeper/ Surveyor	Varroa observed on adult bees (yes/no)	Last Varroa treatment (hive/ compound/ date)	Board clean date	Mite assess date	Count of Mites	Board clean date	Mite assess date	Count of Mites	Board clean date	Mite assess date	Count of Mites

WP1.5.3 Small Hive Beetle Infestation

I Bottero, S Hodge, A Knauer

05/03/19

1. Small Hive Beetle (**SHB**), *Aethina tumida*, are parasites and scavengers of honeybee hives.
2. To save time, the SHB assessment should take place at the **same time** as the ***Varroa*** assessment (WP1.5.2).
3. For PoshBee, the field detection of SHB is based on **visual detection** of **adult** individuals.
4. The adult individual is black, approximately 1/3 of honeybee worker (around 5-6mm), and can be identified by the characteristic club shaped antennae, shape of the pronotum and shape of tibia (see images below). Adults can be found everywhere in the hive, especially on the bottom board.
5. As the SHB has so far only been detected in few European regions, we will only record presence/absence data. The detection of SHB will be made using "**Beetle Blaster"** style traps.
6. A Beetle Blaster (23 cm long and 3.5 cm deep) is a plastic device inserted between frames.
   The trap is designed to fit neatly between outer frames in a hive. The holes in the top of Beetle Blaster are big enough to allow SHBs to enter but cannot be accessed by honeybees.
7. Beetle Blasters can be bought from local beekeeping companies, usually for under €5.
   Similar devices can be bought from online suppliers such as Amazon and Ebay at much lower prices.
8. Before use, 25 ml oil (such as corn or sunflower oil) is added to each trap.
   The traps should not be completely filled to ensure that trapped SHBs cannot reach the top and climb out.
9. **One** trap will be placed in each hive.
   The trap is inserted between the outer frames of the top box of the brood chamber (in some hive systems the brood chamber has two boxes in some only one) (see image below).
10. After several days (a minimum of 3 days is advised) the trap should be removed from the hive and SHB removed by shaking the trap over a clean, white tray.
11. SHB adults can be counted in the field. Data for **each hive** should be recorded in the table below. We will express SHB infestation rates as 'specimens obtained per day'.
12. SHB is present only in one EU Member State (Italy). SHB is a statutory notifiable pest in the European Union, and if adult specimens are detected they **MUST** be sent to your local National Reference Laboratory (NRL) and/or competent authority of the country for identification. Do not send live beetles in the post. Kill them first by keeping them in a freezer overnight or by putting them in 70% ethanol. You should also report the presence of SHB to your local beekeeping association. Seek advice about which other departments in your country should be notified.

References

Neumann P, Evans JD, Pettis JF, Pirk CWW, O Schäfer M, Tanner G, Ellis JD. (2013) Standard methods for small hive beetle research. Journal of Apicultural Research Vol.52 DOI 10.3896/IBRA.1.52.4.19

(Photo from Flickr. Green.thumbs. 2006)

Small Hive Beetle (SHB) results table

		Hive #1			Hive #2			Hive #3		
Site	Beekeeper/ Surveyor	Date traps placed	SHB assess date	Count of SHB	Date traps placed	SHB assess date	Count of SHB	Date traps placed	SHB assess date	Count of SHB
OSR_1										
OSR_2										
OSR_3										
OSR_4										
OSR_5										
OSR_6										
OSR_7										
OSR_8										
APP_9										
APP_10										
APP_11										
APP_12										
APP_13										
APP_14										
APP_15										
APP_16										

WP1.5.4 Presence of *Vespa velutina*

S Hodge, I Bottero

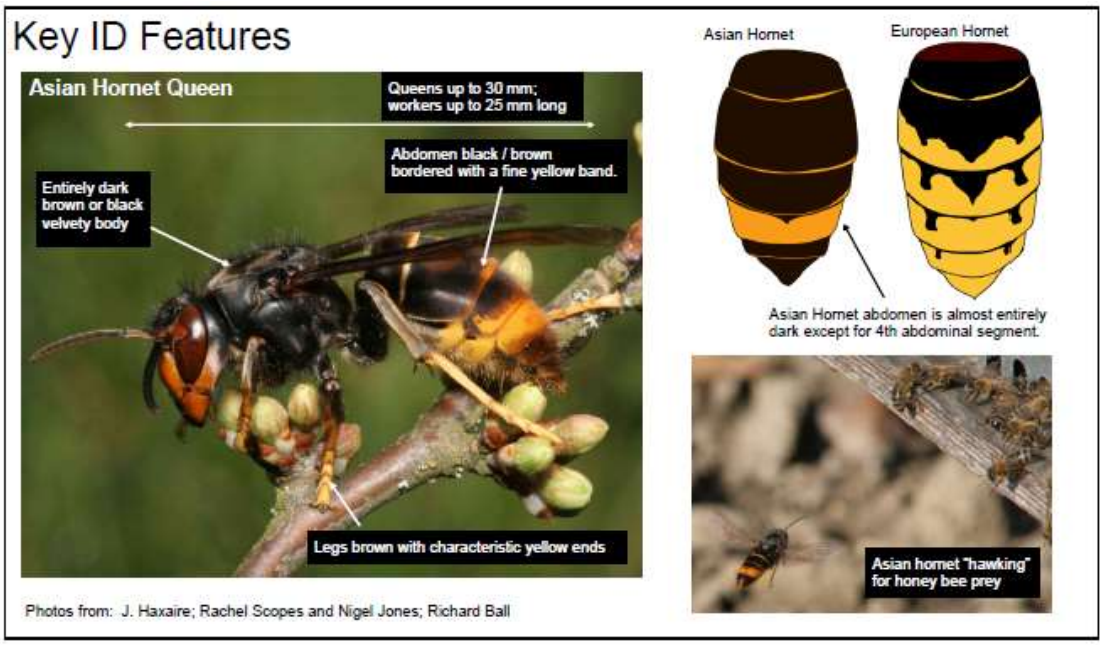
07.03.19

1. The first European record of the Asian hornet ('yellow-legged hornet') *Vespa velutina* occurred in France in 2005; it has subsequently spread throughout mainland Europe and the UK.
2. *V. velutina* is a major predator of bees and contributes to colony losses.
3. EFSA Panel on Animal Health and Welfare (AHAW) suggested the collection of data on the presence of *V. velutina* is recommended in field surveys (EFSA Journal 2016)
4. **The detection of *V. velutina* is notifiable in some countries. Please be aware of your local regulations. Report its occurrence to your beekeepers association immediately.**
5. Workers are approximately 2.5 cm long; colour can be variable, but generally the abdomen is dark and legs have yellow tips.
6. As *V. velutina* is often more common towards the end of summer, and as our field work is relatively early in the year, observations of this species may be rare.
7. We will record the **numbers** of *V. velutina* at **each site** on **each visit** (for up to 5 visits).
8. On each site visit check the entrances to the bee hives for 'hawking' *V. velutina.*
9. Record the date of the visit, and the number of *V. velutina* observed in the table below.
10. Also add the initials of the surveyor to each observation.

References

EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare), 2016. Scientific opinion on assessing the health status of managed honeybee colonies (HEALTHY-B): a toolbox to facilitate harmonised data collection. EFSA Journal 2016;14(10):4578, 241 pp. doi:10.2903/j.efsa.2016.4578

Asian Hornet Alert! Nonnativespecies.org. nationalbeeunit.com
webpage: www.nationalbeeunit.com/downloadDocument.cfm?id=698



Videos

https://www.youtube.com/watch?v=QzeDskBHl8U

https://www.youtube.com/watch?v=fsAbTJ1-YAs

***Vespa velutina* results table**

Site code	Date, Numbers *V.v*, Surveyor	Date, Numbers *V.v*, Surveyor	Date, Numbers *V.v*, Surveyor	Date, Numbers *V.v*, Surveyor	Date, Numbers *V.v*, Surveyor
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

WP1.5.5 Chalkbrood

S Hodge, I Bottero, E Attridge

07.03.19

1. Chalkbrood is a common and widespread disease of honeybees caused by the fungus *Ascosphaera apis* (Jensen et al 2013).
2. Chalk Brood can result in severe reduction of emerging worker bees and colony productivity.
3. The field diagnosis of Chalkbrood is based on visual detection of diseased, mummified brood (commonly known as “mummies”).
4. Beekeepers may diagnose the probable presence of Chalkbrood in a hive based on the presence of the hard, shrunken chalk-like mummies in and around the entrance to the hive. The mummies will be white to grey-black in colour.
5. Infected hives also show a scattered brood pattern or appearance because worker bees will uncap the cells of dead larvae, making mummies clearly visible.
6. We will score Chalkbrood by visual inspection of **all** frames in **each hive.**
7. Familiarise yourself with Chalkbrood symptoms by using the many online resources available.
8. During the inspection, gently shake bees from the frame to allow a full and free view of the brood.
9. Carefully remove each brood frame from the hive, and check both sides for the **presence** of Chalk Brood. Score Chalkbrood as **present** if it is visible on **either** side of the frame.
10. In the results table below, **for each hive at each site**, record the **total number of frames**, and the number of frames where **Chalkbrood is present**.

References

Jensen et al. (2013) Standard methods for fungal brood disease research. J Apic Res 52 doi:10.3896/IBRA.1.52.1.13.

See also beeaware.org.au/archive-pest/Chalk Brood/

(photo by E. Attridge)

Chalkbrood Results Table

			Hive #1		Hive #2		Hive #3	
Site	Date	Beekeeper / Surveyor	Total frames	Chalkbrood frames	Total frames	Chalkbrood frames	Total frames	Chalkbrood frames
OSR_1								
OSR_2								
OSR_3								
OSR_4								
OSR_5								
OSR_6								
OSR_7								
OSR_8								
APP_9								
APP_10								
APP_11								
APP_12								
APP_13								
APP_14								
APP_15								
APP_16								

WP1.5.6 American Foulbrood

S Hodge, I Bottero, E Attridge, M-P Chauzat

07.03.2019

1. American Foulbrood (**AFB**) is a bacterial disease of the brood induced by *Paenibacillus larvae.*
2. The *Apis* larva dies in the cell after the cell is sealed.
3. **AFB is an EU NOTIFIABLE DISEASE and if discovered appropriate action must be taken. Also report your findings to your beekeepers association immediately.**
4. To assess a hive for AFB, first open the hive and clear the bees from the frames by holding the frame inside the hive and shaking firmly.
5. Examine the brood frames to look for 'pepper pot brood' (known also as mosaic brood or patchy brood)
6. Examine all brood cells with **sunken, perforated or discoloured cappings** (Fig 1). If suspect brood cells are detected, open the cells and look for diseased larvae and 'scales'.
7. Turn the frame into the sunlight and look for scales at the bottom of the cells. Scales are harder and difficult to remove than those typically found with European foulbrood.
8. Use a spatula (or match stick/ cocktail stick) to examine the larvae or remaining contents of the cell.
9. If the contents come out 'ropy' or 'stringy' it could be American foulbrood (Fig 2).
10. Infected brood also releases a 'fishy' odour.
11. Sometimes empty cells are present as dead/sick larvae have been removed by workers.
12. Sometimes can see a brow/ red spot or liquid drop in the cell.
13. The suspect sample can be confirmed with a lateral flow device (see below):

a. Diagnostic kits for AFB should be purchased: they will come with detailed instruction for use and interpretation (Fig 4.)

b. Generally, the sick larvae is added to the vial provided, and mixed with reagents provided in the kit. Shake to mix with the solution and dispose one drop of the mix on the lateral flow device. The positive result will normally be expressed as two lines appearing on the device.

c. A test sample can consist of more than one larvae; to save time/ money it is possible to prepare a homogenized sample of multiple larvae and test for both AFB and EFB.

14. Note that AFB could still be present in a brood even though it was not detected by visual inspection (Fig 3).
15. Please fill in the results table for AFB below. Score AFB as detected (or not) in each hive at each site.

See also: https://sites.anses.fr/en/minisite/abeilles/free-access-documents-0

Fig 1. Clear the bees off the frames to examine the brood. Using a match stick examine brood under cappings that are sunken, discoloured or with bite taken out of the corners (Photo E. Attridge)

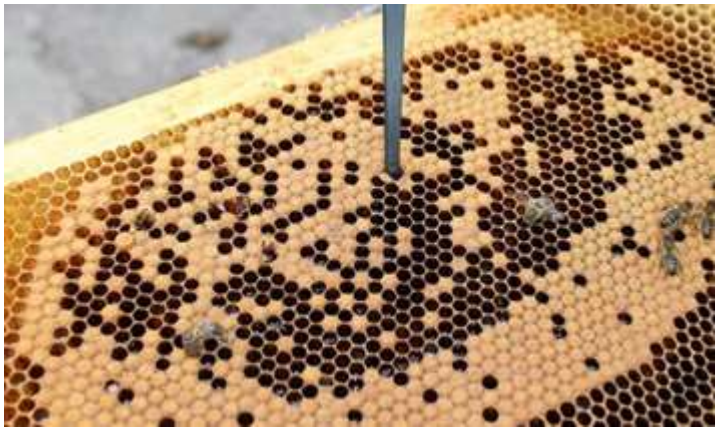
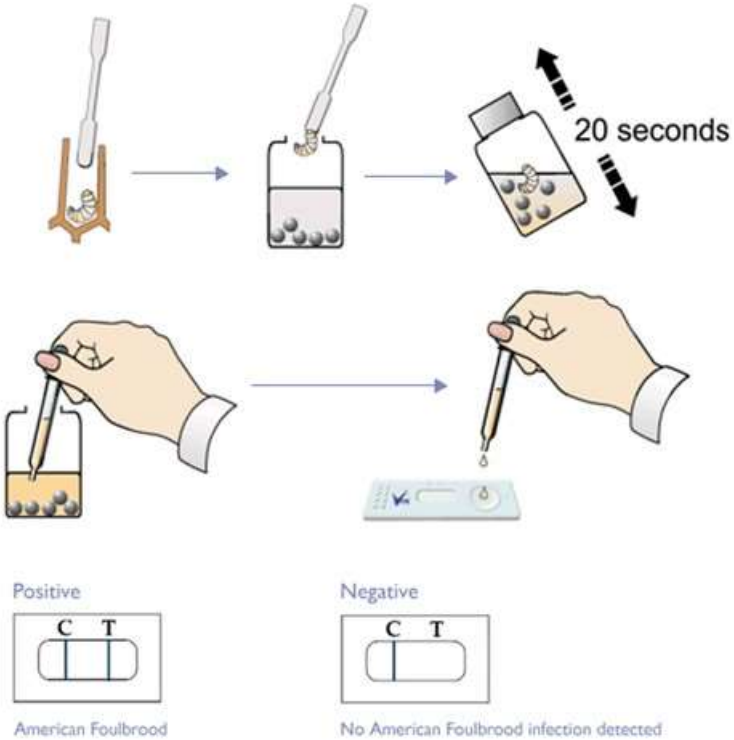


Fig 2. 'Ropy' or stringy contents can be further examined using a lateral flow device for AFB (Photo E. Attridge)

Fig 3. A frame of bees that to the eye doesn't look too bad but had a positive for AFB using a lateral flow device (Photo E. Attridge)

Fig 4. Example of instructions for AFB diagnostic kit from vita-europe.com. Video online. https://www.vita-europe.com/beehealth/products/afb-diagnostic-test-kit/



Results table for AFB. Please indicate whether AFB was detected (1) or not detected (0) in each hive at each site.

DATE	SITE	BEEKEEPER/ SURVEYOR	HIVE 1 AFB	HIVE 2 AFB	HIVE 3 AFB
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

WP1.5.7 European Foulbrood

S Hodge, E Attridge, MP Chauzat

07/03/2019

1. European Foulbrood (**EFB**) is a bacterial disease of the brood caused by *Melissococcus plutonius.* The *Apis* larva is deprived of food as the bacteria obtains nutrition from food fed to the larva.
2. The larva may appear contorted in its cell, and normally dies before the cell is capped. Often the larva loses segmentation.
3. Worker bees sometimes remove the dead larvae and deposit them outside the hive, which can be used as an indicator that EFB is present.
4. **NB: In some countries EFB is a NOTIFIABLE DISEASE and if discovered appropriate action for that country must be taken. Inform your beekeepers association immediately.**
5. The field diagnosis of EFB is based on the visual inspection of brood combs and detection of diseased larvae.
6. To assess a hive for EFB, first open the hive and clear the bees from the frames by holding the frame inside the hive and shaking firmly.
7. The general symptoms of EFB in a colony are:
   - irregular capping of the brood
   - capped and uncapped cells being found scattered irregularly over the brood frame (known as pepper pot brood, mosaic brood, or patchy brood. This symptom can be caused by other problems as well, such as AFB.
8. The colour of affected larvae changes from pearly white to pale yellow.
   Segmentation becomes less noticeable, and the larva becomes fluid and looks 'melted'.
9. **The youngest larvae** that die from EFB cover the bottom of the cell and are almost transparent.
10. **The older larvae** die in strange positions and tend to be flaccid in their cells, twisted or stretched out lengthways. These larvae may be more prevalent towards the periphery of the frame, as a strong colony will quickly clean them out from the centre of the comb.
11. Any suspect larvae should be removed with fine forceps – the gut will be yellow
12. The 'melted' larva should not be as ropey or stringy as with AFB.
13. More advanced symptoms can manifest as further colour changes to brown and greyish black, sometimes leaving a dark scale that is softer and easier to remove with fine forceps than those typically found with American foulbrood (Fig 1). Turn the frame into the sunlight and look for scales at the bottom of the cells.
14. **A suspect sample should be confirmed with a lateral flow device. A test sample can consist of more than one larvae. To save time/ money it is possible to prepare a homogenized sample of multiple larvae and test for both AFB and EFB.**
15. Please fill in the results table below. Score EFB as detected (or not) in each hive at each site.

References

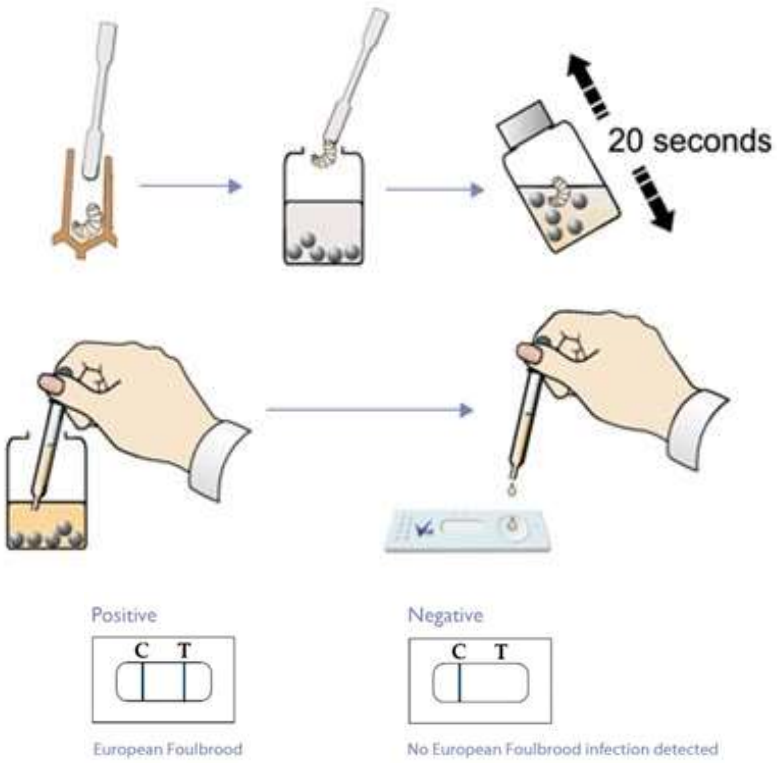
Forsgren E et al. (2013) Standard methods for European foulbrood research, Journal of Apicultural Research, 52:1, 1-14, DOI: 10.3896/IBRA.1.52.1.12

See web pages for images

http://www.nationalbeeunit.com/public/beekeepingFaqs/europeanFoulbroodEfb.cfm

http://beeaware.org.au/archive-pest/european-foulbrood/#ad-image-0

Example of instructions for EFB diagnostic kit from vita-europe.com. Video online.
https://www.vita-europe.com/beehealth/products/efb-diagnostic-test-kit/



Results table for EFB. Please indicate whether EFB was detected (1) or not detected (0) in each hive at each site.

DATE	SITE	BEEKEEPER/ SURVEYOR	HIVE 1 EFB	HIVE 2 EFB	HIVE 3 EFB
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

WP1.5.8 Assessment of the presence of Deformed Wing Virus (DWV)

S Hodge

06.03.19

- We will assess each hive for the presence of Deformed Bee Virus (**DWV**) (or, strictly, just the presence of deformed wings) using information provided by the National Bee Unit (2017).
- In the absence of *Varroa*, deformed wing virus (DWV) persists at low levels in infected colonies without causing any major signs of infection
- In the presence of *Varroa*, DWV causes clinical symptoms in developing pupae, including pupal death and newly emerged bees show **deformed** or **poorly developed wings**.
- Additional symptoms include a **bloated and shortened abdomen**.
- The appearance/extent of deformity depends upon the stage at which individual bees become infected.
- Bees infected as adults appear normal.
- Because bees with DWV cannot fly, colony performance is lowered.
- DWV is also associated with reduced life span, patchy brood and reduced colony populations.
- Although you may not see any signs of DWV in your apiary, it may still be present.
- DWV infection can be confirmed using molecular methods such as PCR
  (ANSES will check for this pathogen)

- Assess each hive for the presence of DWV by lifting out frames and scanning newly-emerged or young workers for the presence of deformed wings
- To save time, the assessment should be performed when carrying out other assessments of bee health, such as chalkbrood, EFB and AFB
- The assessment will be performed **ONCE** towards the end of the trial period.
- You may also check dead bees around the colony to see if they have deformed wings.
- The presence of an experienced beekeeper would be an advantage when performing DWV assessment.
- Record your data in the table provided.

References

The National Bee Unit. 2017. Common Pests, Diseases and Disorders of the Adult Honey Bee. The Animal and Plant Health Agency.


(photo by E. Attridge)

Results table for DWV. Please indicate whether bees showing DWV symptoms (deformed wings; bloated abdomen) were present (1) or not present (0) in each hive at each site.

DATE	LOCATION	BEEKEEPER/ SURVEYOR	HIVE 1 DWV	HIVE 2 DWV	HIVE 3 DWV

WP1.5.9 Assessment of *Bombus terrestris* colony performance and natural enemies

M Rundlöf, S Hodge

08.03.19

Background

We will estimate the performance of *Bombus terrestris* colonies in relation to the varying conditions in the WP1 site network, as well as estimate the infestation of some visible pests. Because the colonies will only be in the field for a limited number of weeks and be terminated at the end of crop bloom, we will only collect basic measures of colony development and reproduction.

This protocol describes how to:

i. assess bumblebee colony growth by weight and worker counts

ii. assess bumblebee colony reproduction by counts of eclosed reproductives, queen cocoons and worker/male cocoons

iii. quantify degree of pest infestation and other inhabitants

Because we are freezing the nests we CANNOT examine emergence of any predators, parasitoids from cocoons or larvae at a later date.

Individual WP1 partners, or small groups of partners, are free to extend the study of various aspects of bumblebee performance once the basic data required by WP1 have been obtained.

This protocol does **NOT** address pathogens and diseases present in *B. terrestris*, because this aspect of the study will be addressed by molecular analysis performed as part of WP2. We will, however, score colonies for the presence/ absence of deformed wing symptoms.

Field work

1. Each of the three bumblebee colonies at each site should be given a unique ID (B1, B2, B3; see WP1.1.2).
2. We will weigh the colonies **THREE** times:
   i) before instalment at the field sites
   ii) during the middle of flowering
   iii) when the colonies are retrieved from the field sites
3. Record the data in **Table 1** below.
4. It is sensible to use **bee suits** during handling of the colonies.
5. Before placement in the field, weigh the individual colony boxes (excluding any outer package or sugar solution provider) and note the **weight** (g) on the attached field datasheet along with SITE ID, COLONY ID and DATE. A chargeable/battery operated scale that can weigh up to 3-5 kg should be a good option.
6. Weigh the colonies again during peak crop bloom, when foragers are collected for analyses. Place the scale in a large **plastic box** during weighing, to give the scale a sturdy surface and reduce the influence of wind during weighing in the field. Note SITE ID, COLONY ID, weight, and DATE on datasheet.
7. Finally weigh the colonies when they are collected from the field for termination.
   Note **weight**, SITE ID, COLONY ID, DATE and TIME on datasheet.

Termination of colonies

1. When the crop has stopped flowering, the bumblebee colonies will be terminated by freezing for collection of pollen stores, sampling of bees for analyses and assessment of the colony performance and health in the lab.
2. It is sensible to use **bee suits** during the collection process.
3. Before collection close the nest entrance/exit.
4. Ideally, colonies should preferably be collected from the field after sunset or before sunrise when most foragers will still be inside the box. Practically, this requirement is unlikely to be possible when visiting multiple sites in the same day.

In this case, it might be only possible to avoid sealing and retrieving *Bombus* boxes in the middle of the day (eg between 10am and 3 pm) when many foragers could be away from the colony.

5. Place the colony in a plastic bag and seal the bag with tape. Label the bag with the SITE ID, COLONY ID, COLLECTION DATE and COLLECTION TIME. Add these data to the table below.
6. Transport as soon as possible to a freezer set to at least -20 C. Place the colonies, still in the marked plastic bags, in the freezer and keep there for at least 24 hours and until the colonies can be processed in the lab.

Lab work - Colony Performance

1. Take the colony out of the freezer and put it on a clean paper on the workbench.
2. Note the SITE ID, COLONY ID and DATE on the lab datasheet.
3. Take a standardized picture of the nest, with a note stating site and colony IDs as well as a ruler on the side.
4. In **Table 2** below, record the numbers of:
   i) adult and emerged workers
   ii) males (drones)
   iii) natal queen
   iv) new queens (gynes)

   Females and **males** are separated by the female's stinger and the male's lack of the same. **Worker** and **queen** females are separated by the larger size of the queens.
   Separate workers into those who were alive during the freezing of the colony and those who had died previously. This distinction is made easiest by checking how dry and "crispy" the bees are, especially the underside of their abdomen.
   The **natal queen**s often have damaged wings and a generally more "worn out" appearance than new queens. Check the status of the natal queen and new queens (live or dead at termination) the same way you do with workers.
5. Scan the workers for signs of DWV. Record presence/absence of DWV in **Table 3** below.
6. Put each caste in a separate plastic tube (e.g. 50 ml Falcon tubes) marked with site id, colony id, caste and status (alive/dead at termination).
7. **At this stage you may want to separate the bee specimens required for WP2 pathogen analysis (WP1.4.4) and bee specimens needed for wing asymmetry and fat body analysis (WP1.6.1**)
8. Remove any wax cover carefully. Forceps and scissors and even razor blades are useful tools here.
9. Take another standardized picture of the nest once the wax cover is removed, including the id note and ruler.
10. Carefully sort through the brood and nest structure and record the number of intact and eclosed
    i) worker/male cocoons
    ii) queen cocoons

    The separation of worker/male and queen cocoons can be done based on the width of the cocoons: usually <12 mm for worker/males and >12 mm for queens in *B. terrestris* (see supplementary figure 1c in Rundlöf et al. 2015).
11. Record the number of
    i) pollen storage cells
    ii) wax cups used for nectar storage.

    Wax cups look like eclosed cocoons with reinforced edges but are made out of wax.

12. At this stage you may want to collect the stored pollen from the storage cells as outlined in WP1.4.5

13. **OPTIONAL 1**: The aim of this option is to get a full picture of the developmental stage and growth of the colony. Open all intact cocoons and note if female or male, alive or failed at termination and if there are signs of parasitism. Count the number of egg and larval clumps and the number of separated larvae, by carefully opening the wax clumps.
14. **OPTIONAL 2**: The aim of this option is to use size of the bees as a quality measure reflecting their environment. Measure and note the inter-tegular distance (ITD) of workers, males and new queens with callipers. Take measurements of 12 individuals of each category, if possible. Note if anything is abnormal with the measured specimen, eg. dead, partially destroyed, or deformed wings.

Lab work - Presence of pests and other inhabitants

15. We will just record presence/absence of the main groups of arthropod predators/ parasites/ kleptoparasites that occur in the *Bombus* colonies (although some of these taxa may be commensals).
16. WP1 partners have good opportunities to expand this area of the study in terms of widening the taxa studied and using species level identifications.
17. In each colony, record presence of
    i) cuckoo bumble bees (eg *Psithyrus*)
    ii) vespulid wasps
    iii) parasitoid wasps
    iv) Diptera/ flies
    v) spiders
    vi) mites
    vii) ants
    viii) wax moth webs
    ix) wax moth larvae (see image opposite)
    x) wax moth cocoons
    xi) wax moth adults

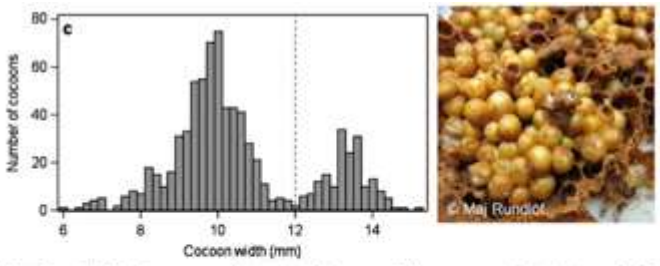
18. Record presence/absence of mould in the colony.
19. Store all invertebrate pest specimens in labelled plastic bags in the freezer for possible future use by you or another WP1 Partner.

Clean up

20. Store brood and nest remains in a labelled plastic box, noting project, year of study and site and colony IDs. Put the material back in the freezer.
21. Weigh the empty colony box after all its content has been removed and note weight on **Table 1**.
22. Clean the workspace and wash all equipment with soap and water and make sure everything that is supposed to be noted and collected have been done so properly.
23. Process the next colony.

References

Rundlöf et al. (2015) Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature 521: 77-80. DOI: 10.1038/nature14420



Extended Data Figure 1 | *O. bicornis* emergence and *B. terrestris* colonies. **a**, Mean (± 95% confidence limits) proportion emergence of *O. bicornis* from cocoons in relation to treatment (control or insecticide seed coating), with higher emergence for males than females (generalised linear mixed model, binomial error distribution, logit link; $F_{1,14} = 14.97$, $P = 0.0017$), no difference between treatments ($F_{1,7} = 0.71$, $P = 0.43$) and no interaction ($F_{1,14} = 0.01$, $P = 0.94$). $n = 8$ fields per treatment, with 12 female and 15 male cocoons at each field. Photos (with permission, Morgan Boch): left, emerged *O. bicornis* cocoon; right, *O. bicornis* female at a trap nests filled with cardboard nest tubes. **b**, Mean (± 95% confidence limits) weight of *B. terrestris* colonies at placement at the fields in relation to treatment (linear mixed model, $F_{1,7} = 0.99$, $P = 0.35$). $n = 8$ fields per treatment, with six colonies at each field. Photos (M.R.): left, *B. terrestris* worker foraging in the oilseed rape; right, house containing three *B. terrestris* colonies. Means and confidence limits in panels **a** and **b** are based on back-transformed, model-estimated least square means. **c**, *B. terrestris* silk cocoon width distribution of all cocoons in four colonies (two from two different control fields and two from two different fields with insecticide seed treatment) initially examined to separate between queen and worker/male cocoons. Dashed vertical line indicates selected cut-off width at 12 mm (the lowest value between the two peaks), with queens larger (or equal) and workers/ males smaller. Photo (M.R.): *B. terrestris* colony under examination.

Additional details on separating female worker and male bumblebees:

The most reliable method to distinguish between workers and males is to check their genitalia. Males often have a larger gap at the end of their abdomen, between the last sterna and terga, and have two clampers that usually is visible in this gap. If these are not visible you have to separate the last sterna and terga carefully with a needle or forceps and check for the clampers inside the abdomen. Workers have a smaller gap and a stinger instead of the clampers. The two also differ slightly in their appearance, males having slightly longer hind legs and a more square shaped end of their abdomen.

Table 1: *Bombus* colony weights

		Installation		Flowering		Retrieval			Empty box	
SITE	COLY	DATE	WGT (g)	DATE	WGT (g)	DATE	TIME	WGT (g)	DATE	WGT (g)
OSR_01	B1									
OSR_01	B2									
OSR_01	B3									
OSR_02	B1									
OSR_02	B2									
OSR_02	B3									
OSR_03	B1									
OSR_03	B2									
OSR_03	B3									
OSR_04	B1									
OSR_04	B2									
OSR_04	B3									
OSR_05	B1									
OSR_05	B2									
OSR_05	B3									
OSR_06	B1									
OSR_06	B2									
OSR_06	B3									
OSR_07	B1									
OSR_07	B2									
OSR_07	B3									
OSR_08	B1									
OSR_08	B2									
OSR_08	B3									
APP_09	B1									
APP_09	B2									
APP_09	B3									
APP_10	B1									
APP_10	B2									
APP_10	B3									
APP_11	B1									
APP_11	B2									
APP_11	B3									
APP_12	B1									
APP_12	B2									
APP_12	B3									
APP_13	B1									
APP_13	B2									
APP_13	B3									
APP_14	B1									
APP_14	B2									
APP_14	B3									
APP_15	B1									
APP_15	B2									
APP_15	B3									
APP_16	B1									
APP_16	B2									
APP_16	B3									

Table 2. *Bombus* colony performance

SITE	COLY	DATE	Workers	Males	Natal Q	New Q	Wor/Ma Cocoons Intact	Wor/Ma Cocoons Eclosed	Queen Cocoons Intact	Queen cocoons Eclosed	Pollen Cells	Wax Cups
OSR_01	B1											
OSR_01	B2											
OSR_01	B3											
OSR_02	B1											
OSR_02	B2											
OSR_02	B3											
OSR_03	B1											
OSR_03	B2											
OSR_03	B3											
OSR_04	B1											
OSR_04	B2											
OSR_04	B3											
OSR_05	B1											
OSR_05	B2											
OSR_05	B3											
OSR_06	B1											
OSR_06	B2											
OSR_06	B3											
OSR_07	B1											
OSR_07	B2											
OSR_07	B3											
OSR_08	B1											
OSR_08	B2											
OSR_08	B3											
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APP_09	B3											
APP_10	B1											
APP_10	B2											
APP_10	B3											
APP_11	B1											
APP_11	B2											
APP_11	B3											
APP_12	B1											
APP_12	B2											
APP_12	B3											
APP_13	B1											
APP_13	B2											
APP_13	B3											
APP_14	B1											
APP_14	B2											
APP_14	B3											
APP_15	B1											
APP_15	B2											
APP_15	B3											
APP_16	B1											
APP_16	B2											
APP_16	B3											

Table 3. Presence/absence *Bombus* colony pests [WM - wax moth]

SITE	COLY	DATE	DWV	WM webs	WM larvae	WM cocoons	WM adults	Cuckoo bees	Flies	Wasps	Parasitoid	Ants	Spiders	Mites
OSR_01	B1													
OSR_01	B2													
OSR_01	B3													
OSR_02	B1													
OSR_02	B2													
OSR_02	B3													
OSR_03	B1													
OSR_03	B2													
OSR_03	B3													
OSR_04	B1													
OSR_04	B2													
OSR_04	B3													
OSR_05	B1													
OSR_05	B2													
OSR_05	B3													
OSR_06	B1													
OSR_06	B2													
OSR_06	B3													
OSR_07	B1													
OSR_07	B2													
OSR_07	B3													
OSR_08	B1													
OSR_08	B2													
OSR_08	B3													
APP_09	B1													
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APP_11	B1													
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APP_13	B1													
APP_13	B2													
APP_13	B3													
APP_14	B1													
APP_14	B2													
APP_14	B3													
APP_15	B1													
APP_15	B2													
APP_15	B3													
APP_16	B1													
APP_16	B2													
APP_16	B3													

WP1.5.10 Assessment of *Osmia bicornis* performance and exposure to natural enemies

M Albrecht, S Hodge, A Knauer, M Rundlöf

29.03.19

1. In this protocol, the methodologies of assessing levels of natural enemy attack, basic performance and reproductive success are described. **MEASUREMENT OF REPRODUCTIVE SUCCESS IS OPTIONAL FOR INTERESTED PARTNERS.**
2. *Osmia bicornis* can be attacked by a series of natural enemy species (predators or parasites /parasitoids), which can contribute mortality or adverse sublethal effects on offspring development and fitness (e.g. through deprived food provisions).
3. *Osmia* are understudied compared to Bombus and *Apis* and any data we obtain for *Osmia* will likely be novel and highly valuable. Therefore, we will try to obtain some basic assessment on the occurrence of *Osmia* natural enemies over the whole site network. Interested partners can further measure reproductive success.
4. This protocol shall describe how to
   (i) measure basic performance of *Osmia bicornis* across all WP1 sites
   (ii) identify the key natural enemy taxonomic groups attacking *O. bicornis* and their contribution to offspring mortality across all WP1 sites.
   (iii) assess *Osmia* reproductive success across the sites of interested partners.
5. This protocol does **NOT** address pathogens and diseases present in *O. bicornis*: this aspect of the study will be addressed by molecular analysis performed as part of WP2.

OPTION 1: Basic performance and natural enemies – protocol for all partners

Field work

1. Each of the three *Osmia* trap nests at each site should be given a unique ID (O1, O2, O3; see WP1.1.2). We suggest that if the nests are attached to a single post, the nests are labelled from O1-O3 from top to bottom.
2. Before releasing the bees in the field, incubate 60 randomly selected cocoons from your stock and record the sex ratio in the data sheet below. The 10 females which are required for pathogen analysis can be taken from these individuals.
3. About two weeks after emergence of bees at the sites, remove all remaining cocoons from the trap nests. This ensures that natural enemies present in the delivered cocoons are not released into the natural habitats.
4. Record the number of carboard tubes you removed from each trap nest for pollen collection (Table 1).
5. At the end of the flowering period of the focal crops, place a fine-meshed piece of netting fabric or mesh over the front of each trap nest with strong outdoor tape. Record this date for each site in Table 1. This mesh will exclude further nesting by bees and entrance of natural enemies from the nests/ cardboard tubes.
6. If possible, leave the sealed trap nests on site for at **least four more weeks** untouched in the field to allow larvae to develop and avoid early-stage larvae falling off the pollen provisions when handling/transporting the nests (which can cause significant mortality).
7. Trap nests should then be collected and stored at a shaded outdoor location under ambient conditions that protects them from sun (i.e. excessive heat), rain and against potential predators until full maturation of the adults in the cocoons for a further 3 months (approximately end of September/mid October). Whether the bees in the cocoons are already fully developed and can be transferred into cold storage has to be checked by opening 10 cocoons.
8. After this period, individually labelled trap nests will be brought back to the lab to be processed.

Lab work

1. Beginning of October remove the netting/mesh of each nest in a mesh cage to capture any pests/ insects that fly out of the tube. Label and store these insects in a plastic bag in a freezer.
2. Record how many sealed and unsealed tubes are in each trap nest (Table 1).

3. Remove the cardboard tubes from each trap nest.
4. Select **30 sealed cardboard tubes per site** or, if you have less, as many as possible. The tubes should be selected in equal proportions from the three trap nests if possible (10 per trap nest). Number these tubes from 1 to 30 and label them with COUNTRY_CROP_SITE_TRAP NEST_TUBE NR (e.g. CHE_APP_02_O2_28).
5. Carefully open these 30 tubes without damaging pupae/killing offspring of bees or natural enemies. This can be done by using a suitable metal tube with a slit; the tube prevents the cutter from cutting to deep.
6. For each opened carboard tube record (in Table 2):
   (i) total number of cells
   (ii) cells with only pollen (no egg visible)
   (iii) cells with a dead larva
   (iv) cells with a pupa
7. Transfer each carboard tube to a glass tube of appropriate length and diameter (one glass tube per carboard tube). Close with a piece of cotton to avoid escape of emerging insects.
   Label all glass tubes with COUNTRY_CROP_SITE_TRAP NEST_TUBE NR.
8. Store tubes for 1 weeks at 10(±2)°C and relative air humidity of 60-80 %.
   Then store in a cool room at roughly 2 (±2) °C and relative air humidity of 60-80 % until mid-March 2020 (hibernation).
9. To bring pupae out of hibernation, store nests for 1 weeks at 10(±2)°C and relative air humidity of 60-80 % and then at 22 (± 2) °C and relative air humidity of 60-80 % to let adult bees and any natural enemies emerge.
10. Collect all specimens that emerge per tube in some suitable vial. Record data on these specimens in Table 3. Identification keys for bees and natural enemies will be provided by the end of 2019.
11. Identify adult bees to species level and record if they are *O. bicornis*.
12. Record the total number of *O. bicornis* bees and the number of females and male *O. bicornis.*
13. Emerging natural enemies will counted and identified to appropriate taxonomic levels.
14. Record the number of pupae that have not hatched and do not show any signs of parasitation/predation.
15. Retain all specimens as these may be identified to species level at a later date.
16. **Send ≥10 *O. bicornis* offspring (≥5 female, ≥5 male) per trap nest to Umons for the analysis of wing asymmetry (WP1.6.2)**

OPTION 2: Reproductive success – for interested partners

Field work

1. Reproductive success will be measured as number of offspring per released female (in one trap nest). Additionally offspring weight and sex ratio will be measured.
2. To measure reproductive success makes only sense for countries that have **low** colonization by natural populations: if all or almost all (e.g. more than approx. 90%) of cardboard tubes at a site are occupied by nesting bees (i.e. cardboard tube sealed) reproductive success cannot be assessed properly (except if sealed cardboard tubes can be regularly exchanged by new empty ones during the nesting period).
3. Few days before flowering, fix all trap nests **on one pole** facing the same direction (south-facing as specified in protocol 1.2.4). This will ensure similar attractiveness of trap nests for *Osmia* females and therefore similar numbers of nesting initiation per trap nest.
4. Give each of the three *Osmia* trap nests at each site a unique ID: **SITE_O1, SITE_O2, SITE_O3 from top down** (see WP1.1.2).
5. Collect all destructive samples (collecting bees and pollen stores) from the upper two trap nests. To collect bees (pathogen, gut microbiota etc.) you need to capture them when they leave the tubes to make sure they are nesting in one of the upper two trap nests. **IMPORTANT: If you cannot get enough bees or pollen stores for WP1.4.4/WP1.4.5/WP1.6.1/WP1.6.2 from the upper two trap nests, you need to use the third trap nest for these samples –** in this case reproductive success of *O. bicornis* cannot be measured at this site. **Measuring reproductive success has lower priority compared to all other measurements.** Be also aware, that collecting samples from only two trap nests will increase your sampling time.
6. Follow step 3 to 8 from the "Basic performance and natural enemies – Field work" protocol above.

Lab work

1. **The following steps only refer to the trap nest O3 per site (i.e. the lowest one, the trap nest reserved for reproductive success measurement, see above).** The other two trap nests should be processed as described above in the "Basic performance and natural enemies" protocol.
2. After removal of netting/mesh from trap nests in September, remove **all** cardboard tubes from the O3 trap nest (lowest one of three attached to the pole). In O1 and O2 an additional 20 tubes (10 per trap nest) are used for assessment of natural enemies (see above).
3. Carefully open **all** tubes without damaging pupae/killing offspring of bees or natural enemies. This can be done by using a suitable metal tube with a slit; the tube prevents the cutter from cutting to deep.
4. Follow step 6 to 15 from the "Basic performance and natural enemies– Lab work" protocol above.
5. Weigh all the emerged bees and record their weight and sex in Table 4.
6. **Send ≥10 *O. bicornis* offspring (≥5 female, ≥5 male) from this trap nest to Umons for the analysis of wing asymmetry (WP1.6.2).**

Sex ratio in stock:

Date: ___________

Number of females: ___________

Number of males: ___________

Table 1: *Osmia* trap nest data

SITE	TRAP NEST	DATE NEST COVERED WITH MESH	No. OF TUBES REMOVED FOR POLLEN	No. OF SEALED TUBES IN TRAP NEST	No. OF UNSEALED TUBES IN TRAP NEST

Table 2: *Osmia* nest tube data

SITE: _____

TRAP NEST NO	TUBE NO	TOTAL CELLS	CELLS WITH ONLY POLLEN	CELLS WITH DEAD LARVAE	CELLS WITH PUPAE

Table 3: Second generation basic performance and natural enemies

SITE: ____

TRAP NEST NO	**TUBE NO**	**Number of alive *O. bicornis* males**	**Number of alive *O. bicornis* females**	**Number of alive bees from other species**	**Natural enemy - taxonomic group 1**	**Number of specimens in group 1**	**Natural enemy - taxonomic group 2**	**Number of specimens in group 2**	**Number of intact, unhatched pupae**

Table 4: Second generation sex and weight (record data for each hatched individual bee per line)

SITE: _____

TRAP NEST NO	CARDBOARD TUBE NO	SEX	WEIGHT

WP1.6.1 Field collection of bee haemolymph for WP9

P Bulet, K Arafah, D Askri, S Voisin, S Hodge

04.03.2019

1. **Aims:** Haemolymph is the circulating fluid in invertebrates, analogous to blood in humans.
   The aim of this process is to mimic human blood analyses for health monitoring of bees.
2. **Brief details on the technique:** We will use MALDI-BeeTyping® which is a novel tool inspired by MALDI BioTyping for monitoring bee health based on haemolymph analyses developed by BioPark. This procedure was established based on previous analyses on *Drosophila* haemolymph (Uttenweiler Joseph et al., Proc. Natl. Acad. Sci. USA; 1998) performed by Philippe Bulet in his former laboratory (Strasbourg, France).
3. **Method:** We will collect haemolymph from **foraging bees** of **each species** (*Apis*, *Bombus*, *Osmia*) from **each WP1 field site.** Collect samples during the **late flowering** period, and between 10am and 4pm when the bees are active. Use a mixture of bees from each of the three hives / nests / colonies at each site. Record the data for each **individual** bee on the sample summary sheet for each site.
4. Minimum requirements for haemolymph: 5 *Apis* per **hive** (15 in total per site)
   5 *Bombus* per **site** (5 in total per site)
   5 *Osmia* per **site** (5 in total per site)

   If there is time and specimens available, **extra samples** of **all species** would be beneficial.
5. Where possible, collect the samples for all three species from the same site on the same day.
   However, for some species, such as *Osmia*, samples may need to be accumulated over different sampling visits. It is estimated that this collecting could take around **2.5 person hours**.
6. We will examine the haemolymph of **individual** bees, so haemolymph samples should be retained in separate tubes, and **NOT pooled** for each site.
7. The **bodies** of the bees used for haemolymph collection will be **retained** in **individual** tubes and sent to **Biopark** (these bodies might later be sent to Anses for pathogen analysis; see WP1.4.4).
8. The tubes come in matched pairs - make sure the haemolymph and body from the same specimen go into one pair of sample tubes. **Red tubes - haemolymph**; **Black tubes - bodies.**
9. There will be **THREE bags** for each site. These bags represent the samples collected from each species of bee. Label accordingly. Put both the **red** and **black** tubes for each species in the appropriate bag.
10. In order to minimise the variability of haemolymph samples due to the experimenter, all the haemolymph must be collected by the **same experimenter(s)**
11. The **sample summary sheet** should be completed with meta data related to each individual bee.
    The specimen code number is obtained from the barcode printed on the tubes.
    Each country will receive a set of sample tubes with unique numbers.
12. Detailed instructions of how to obtain haemolymph are provided below.

Required materials and equipment

Clean water for capillary wash after use	
Ice cubes or freezer blocks	 
Haemolymph collection kit	
Forceps (Provided with the kit)	

Kit content checklist

Quantity	Name	Use	Received?
1	Silicon collector tube (80 cm)	To connect the filter tip to the capillary holder	
5	Filter tip 1000 µL	To blow on the capillary	
1	Capillary holder	To hold the capillary	
Box of 10 (enough for 200 samples)	In-house capillary glass ready for use	To receive haemolymph sample	
Number of ordered tubes with red marks	Coated microcentrifuge tubes 1.5 mL	To collect haemolymph sample	
Number of ordered tubes with black marks	Standard microcentrifuge tubes 1.5 mL	To store bodies of bees used in haemolymph extraction	
2	Bee holder	To hold the bee	
1	Freezer gel packs	To maintain the cold condition	

NB1: The silicon collector tube length (80 cm) could be adjusted also according to the experimenter

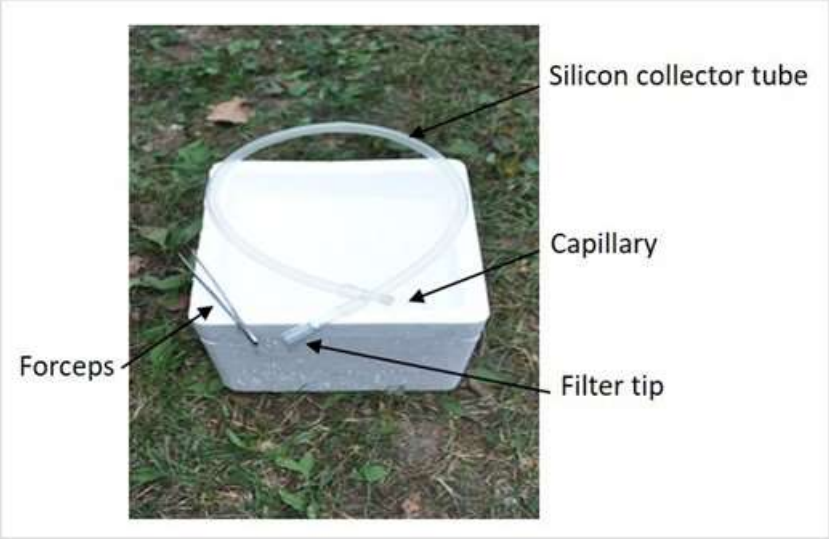
NB2: The 3 cm tube used to hold the bee is used according to the bee size (eg larger size for Bombus)

NB3: When the kit arrives place sample tubes in the freezer to protect the inner coating.

Haemolymph collection - Standard Operating Procedure

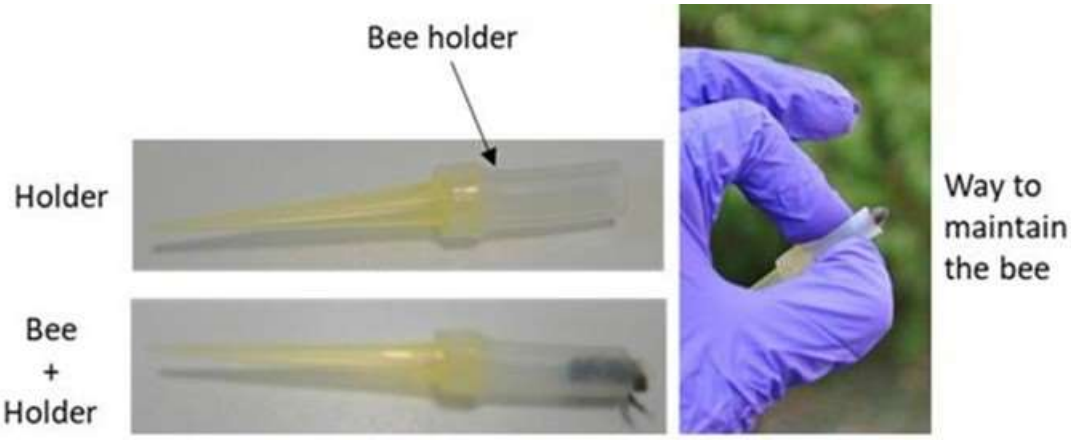
1. **Experiment preparation:** In the field, prepare the required material (Collection kit, forceps, ice, coated microcentrifuge tubes) for hemolymph collection

⚠ Keep the coated microcentrifuge tubes on ice/chilled during the experiment time



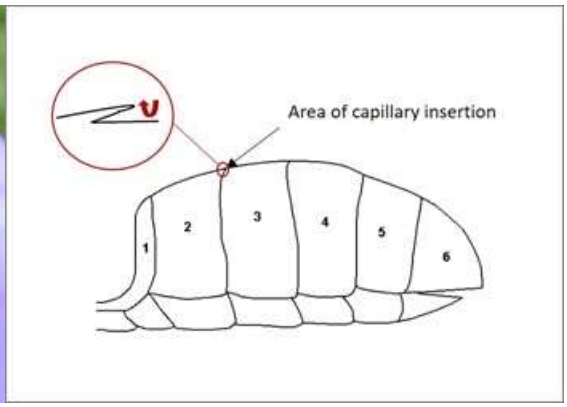
2. **Bee Hold:** Catch the bee by the front legs from the hive box with soft forceps.
Do NOT kill or anesthetize or decapitate the bee before haemolymph collection. DO NOT freeze the bees before haemolymph collection. If needed, you may just cool down the bees in the fridge for maximum 2-3 minutes.
Insert the bee inside the bee holder, the head and the thorax should be placed inside until the first tergum and maintain the bee with the index finger and tighten a little bit the silicon tube between index and thumb. Trap the wings under the abdomen

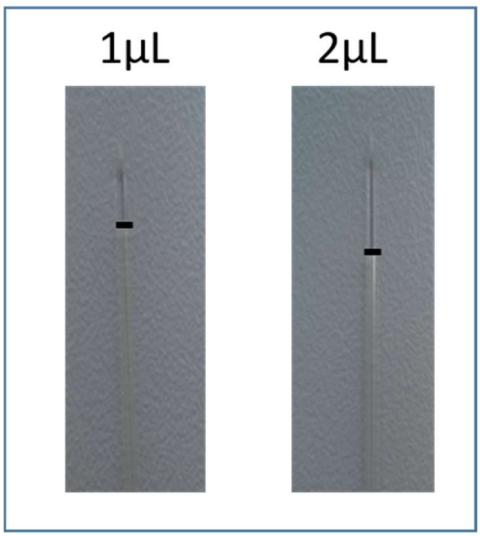
⚠ Avoid that the bee stings in the holder; this could contaminate the sample with venom.



3. **Haemolymph draw:** Insert the capillary dorsally under the second tergum of the abdomen (in the middle horizontally) (see the picture below).
The haemolymph will be drawn out by capillarity, otherwise you can aspirate slowly and carefully through the filter tip

4. ⚠ Aspirate gently and slowly to draw out the fluid; verify the level of the liquid inside the capillary simultaneously. A volume **NOT LESS than 1µL** per bee (ideally 1-2 µL, no problem if you could draw more) is required for proteomic analyses.





5. **Haemolymph quality requirement:** Verify the haemolymph appearance; it should be from transparent to slightly yellow. If it is cloudy yellow or brown this means that your sample is contaminated by the intestinal contents and should be rejected. A new sample collection is required.

6. **Haemolymph transfer to the microcentrifuge tube:** Blow slowly in the filter tip to eject the drawn haemolymph from the capillary **<u>at the bottom</u>** of the coated microcentrifuge tube.

 If you remark that after haemolymph deposit in the tube, that the quality is not acceptable, you can just wash the tube with water and reuse it again.

7. **Haemolymph storage:** Keep the tube on ice and then store it in a freezer (-18 to -16°C) before sending it to BioPark-Archamps (France).

8. **Take off the bee from the holder** and place **t**he **body** in the appropriate **pre-labelled tubes** that have been supplied. Place the bodies in the chilled container and store in a freezer when possible. If the bee stings in the holder, remove the venom sting sac and wash the holder with clean distilled water.

9. **Capillary wash:** Between each sample collection, wash the capillary 3 times with clean water (eg distilled water or bottled water). **NOT TAP WATER.**
You will be supplied with **10 capillaries** for each site.
If the wash is done correctly, one capillary could be used to collect 20 samples.
If you break or spoil all the capillaries then contact Biopark and ask for more.

10. **Change capillaries between species**. There is no need to change capillaries between specimens of the same species.

11. Repeat this process until you have the required number of clear and acceptable samples per site from each bee species

WP9 haemolymph collection sheet.

Please fill in the details below **FOR EACH SITE**

SITE CODE:_____

Species	sample	Bar code	Date	Collector(s)	Hive/nest/colony
Apis	**1**				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
	15				
Bombus	**1**				
	2				
	3				
	4				
	5				
Osmia	**1**				
	2				
	3				
	4				
	5				
Extras	**1**				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				

WP1.6.2 Collecting bee samples for wing asymmetry, fat bodies, gut microbiota

S Hodge, D Michez, O Schweiger

08/03/2019

General procedure

1. As additional measures of bee health and 'fitness', we will assess wing asymmetry, fat bodies and gut microbiota of our sentinel bees.
2. We will only collect these specimens if we have fulfilled the other requirements of WP2
3. At each site, adult insects of the **THREE** bee species (*Apis mellifera*, *Bombus terrestris* and *Osmia spp*.) will be used.
4. The sampling will be performed at various times during the field season. To save time try to collect these bees when collecting specimens for other WP2 protocols (see WP1.4.4)
5. Fill in the **SAMPLE SUMMARY** sheets for each species with details of **site**, **date**, **collectors**, and **quantities** of each species obtained.

Gut microbiota

6. For each **SITE**, collect:

   10 foraging ***Apis***
   5 foraging ***Bombus***
   5 foraging ***Osmia***.

7. Collecting should be performed as described for the WP2 bee collecting protocol **(WP1.4.4)**.
8. These bees should be placed in plastic bags and labelled accordingly, with **SITE_SPECIES_GUT_DATE**.
9. The samples should be stored in a **CHILLED** container and placed in a **FREEZER** as soon as possible.
10. Where necessary, these animals can be collected at the very end of the field sampling, when all other sampling is completed, and we have enough specimens for the other WP2 requirements (especially *Osmia*).

Wing asymmetry and fat bodies

11. The level of wing asymmetry is a function of stress experienced by the developing bee larvae.
12. ***Apis* samples.**
    Collect **20 young NON-foraging** *Apis* from inside **each hive**.
    Collect these bees at the end of the sampling season.
    Process these bees as above: **LABEL**, **CHILL**, **FREEZE**.
    Add **WA** to the label (wing asymmetry): **SITE_HIVE_WA_DATE.**
    These samples could be obtained when collecting the internal workers for WP2 at the end of the flowering period (see WP1.4.4)

13. ***Bombus* samples**
Obtain these samples at the end of the trial, when the *Bombus* nests have been brought in from the field sites and frozen.
Collect **10 young-looking** *Bombus* females **per nest**.
Label with **SITE_COLONY_WA_DATE**

14. (the samples used for pre-screening of pathogens by ANSES will also be sent to UMONS for wing asymmetry measurements)

15. ***Osmia* sample**
    we will need to wait until the following year to collect *Osmia* adults that have developed under the different conditions provided by the site network.
16. Obtain these samples after the *Osmia* nests have been collected in from the field and dissected (for pollen stores, parasites etc WP1.5.10)

Retain at least **10** pupae per trap nest and store in a suitable rigid container.
These pupae will need to be retained until the adults emerge the following year (see WP1.5.10 for details on maintenance of pupae).
When the adults emerge, store in plastic bags, **LABEL**, **FREEZE**.
Label these samples with **SITE_NEST_WA_DATE**.

Postage

17. The **bee samples** for **gut microbiota** will be sent to Oliver Schweiger at **UFZ**.
18. The **bee samples** for **asymmetry** will be shipped to Denis Michez at **UMons.**
19. Care should be taken to maintain the cold conditions and to avoid crushing samples (use rigid packaging). Packages will be posted respecting IATA procedure requirements (UN 1845).
20. To reduce shipment costs, each partner is recommended to organise the shipment when **ALL** the samples of **each species** are collected.

GUT MICROBIOTA: Summary of **bee specimens** collected for **gut microbiota** analysis

			Numbers collected		
Site code	**Date**	**Collector(s)**	***Apis***	***Bombus***	***Osmia***
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

WING ASYMMETRY

Summary of **Non-foraging *Apis*** specimens for **fat bodies and asymmetry analysis**

			Numbers collected		
Site code	**Date**	**Collector(s)**	**A1**	**A2**	**A3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Summary of **Non-foraging *Bombus*** specimens for **fat bodies and asymmetry analysis**

			Numbers collected		
Site code	**Date**	**Collector(s)**	**B1**	**B2**	**B3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					

Summary of *Osmia* pupae collections

			Numbers collected		
Site code	**Date**	**Collector(s)**	**O1**	**O2**	**O3**
OSR_1					
OSR_2					
OSR_3					
OSR_4					
OSR_5					
OSR_6					
OSR_7					
OSR_8					
APP_9					
APP_10					
APP_11					
APP_12					
APP_13					
APP_14					
APP_15					
APP_16					